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(54) Title: METHOD ENABLING READMINISTRATION OF AAV VECTOR VIA IMMUNOSUPPRESSION OF HOST

(57) Abstract

The present invention is directed to a method for providing AAV mediated gene therapy to a patient, comprising administering to a patient a replication-defective adeno-associated virus particle which infects a cell in the patient, the particle having therein a gene encoding a protein needed by the patient, the gene being operatively linked for expression in the cell, and at about the time of above-administering step, also administering to the patient an immunosuppressant that suppresses the patient's humoral immune response. The present invention is also directed to pharmaceutical compositions comprising the above described adeno-associated virus and humoral immuno-suppressant in a pharmaceutically acceptable carrier. Examples of proteins expressed by the above-described vectors include erythropoietin, thrombopoietin, human growth factor, leptin, Factor VIII, Factor IX, Factor Xa and the like.

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METHOD ENABLING READMINISTRATION OF AAV VECTOR VIA IMMUNOSUPPRESSION OF HOST

BACKGROUND

A. Field of the Invention

The present invention is directed to a method for providing a somatic gene therapy. In particular, the present invention is directed to a method for somatic gene therapy, particularly in humans, that comprises administering an adeno-associated viral vector encoding the gene of interest and an immunosuppressant. The present method is useful because it allows for expression of a gene encoded by the AAV vector without inducing a neutralizing immunoresponse.

B. Background of the Invention

All of the viruses proposed for gene therapy, including for example, retroviruses, adenoviruses, herpes viruses and adeno-associated viruses, express proteins recognized as foreign in their mammalian hosts. However, some viral vectors express more foreign proteins than others and are thus, more antigenic. For example, a retroviral vector is an integrating RNA-based vector, which requires expression of both a wild-type reverse transcriptase and integrase to obtain ultimate expression of the recombinant gene. An adenoviral vector is a non-integrating DNA-based vector. However, it still requires the expression of many of its proteins in order to obtain expression of the recombinant gene. Thus, in a patient previously exposed to the wild-type virus of the viral vector, an immune response is generated that would destroy any cells infected by the viral vector. To circumvent this problem, some viral vectors are prepared by modifying a wild-type virus that is selectively pathogenic to a species other than its intended target. For example, the envelope protein of wild-type Moloney murine leukemia virus (MoMLV), whose normal host is a mouse, has been

modified to be amphotropic, and thus capable of infecting other non-mammalian species. Such modifications of viral envelope proteins are extremely laborious. Accordingly, it would be desirable to provide an alternative method of gene therapy that does not require genetic alteration of the specificity of a species-specific virus.

Viral vectors can be either integrating (*i.e.*, requires integration into the host cell DNA to be expressed) or non-integrating. An integrating viral vector provides the prospect for long term gene expression. In contrast, a non-integrating vector provides for short-term gene expression. Accordingly, it is an object of the present invention to provide a method for somatic gene therapy to a patient, wherein the vector integrates into the DNA of the patient's host cell to provide long term somatic gene expression

AAV vectors are single-stranded linear DNA integrating vectors that are non-pathogenic and can infect both dividing and non-dividing cells. The AAV genome, as exemplified by AAV-2, contains two inverted terminal repeats (ITRs) at opposing ends of the virus that are 145 bp long. Gerry *et al.*, (1973) *J. Mol. Biol.79:* 207-225; Kozcot *et al.*, (1973) *PNAS USA 70*: 215-219; and Lusby, *et al.*, (1980) *J. Virol. 34*: 402-409. Each repeat can form a T-shaped hairpin structure which is composed of two small palindromes flanked by a larger palindrome. The AAV coding region, which is between the two ITRs, is divided into three regions: *rep, lip* and *cap*. The function of the *rep* region is to encode for four non-structural proteins that regulate of AAV DNA replication and expression. The function of the *cap* region is to code for the three structural proteins: VP1, VP2 and VP3 of the capsid. AAV preparations are stable and can be produced at high titers ($> 10^{12}$ particles/ml). *See* for example, Flotte & Carter (1995) *Gene Ther.*2, 357-362 and Samulski, (1989) *J. Virol. 63,* 3822-3828. There are recent reports demonstrating long term expression of transgenes following delivery of AAV vectors into lung, liver, muscle, heart and brain. *See* Flotte, (1993) *Proc. Natl. Acad. Sci. USA* **90,** 10613-10617; Koeberl, (1997)

Proc. Natl. Acad. Sci. USA **94**, 1426-1431; Fisher, (1997) *Nat. Med.* **3**, 306-312; Xiao, (1996) *J. Virol.* **70**, 8098-8108; Kaplitt, (1994) *Nat. Genet.* **8**, 148-154; and Kaplitt, (1996) *Ann. Thorac. Sur.* **62**, 1669-1676. Likewise, injection of rAAV vector into skeletal muscle has been shown to lead to persistent, high level expression of transgenes. *See* Fisher, (1997) *Nat. Med.* **3**, 306-312 and Xiao, (1996) *J. Virol.* **70**, 8098-8108.

Unfortunately, however, numerous experiments have demonstrated that after a single intramuscular injection of a rAAV vector, readministration of the rAAV vector does not lead to expression of the recombinant protein, even if it encodes a different recombinant protein. Gene delivery experiments performed with AAV vectors demonstrate that the intracellularly expressed AAV proteins elicit strong cell-mediated immune responses that eliminates the transduced cells. Even when a replication-defective AAV virion lacks all virally encoded genes, the virion is encapsulated in the AAV capsid proteins that are responsible for the entry into the cell and transport of the packaged DNA to the nucleus. Because AAV viruses are relatively ubiquitous and non-pathogenic, a majority of the population of animals and humans has been exposed to one or more of the seven serotypes of AAV and has developed an immune response thereto. This response would neutralize any attempted gene therapy that employed an AAV vector of the same serotype as the immunizing strain. Accordingly, it is an object of the present invention to develop a method for administering an AAV vector to a patient in need of somatic gene therapy, wherein protein expression would not be neutralized by the patients' immune system.

SUMMARY OF THE INVENTION

The Applicants have discovered that a patient that has developed an immune response to one or more adeno-associated viruses (AAV) may none the less be recipient for AAV vector-mediated gene therapy, if prior to, during or within a short time after the time of administering an rAAV vector particle, the humoral arm of the immune system of the patient is transiently immunosuppressed. Thus in one aspect, the present invention is directed to a method for obtaining *in vivo* expression in a patient of a therapeutic agent encoded by a gene contained within an AAV vector, the patient suspected of having an immune response to AAV, the method comprising administering to the patient in need of the therapeutic agent a replication defective recombinant AAV particle (virion) having a gene encoding the therapeutic agent; and before, during or within a short period after administering the AAV vector, transiently immunosuppressing the humoral immune response of the patient to obtain expression of the therapeutic agent. In the above method, the therapeutic agent is a protein, polypeptide, antisense RNA or a ribozyme. Preferably, the therapeutic agent is a protein or a polypeptide.

By the phrase "transiently immunosuppressed" or "transient immunosuppression," as used herein, is meant that the patients' humoral (antibody producing) immune response has been reduced when compared to the patient's immune response in the absence of the immunosuppressive treatment. The transient immunosuppression of the patient's humoral immune system is accomplished by administering to the patient a pharmaceutical composition comprising For other art recognized humoral immunosuppressive agent or a combination thereof. Preferably, the immune system of the patient is transiently immunosuppressed by administering to the patient a pharmaceutical composition comprising an antibody that is an anti-CD4, anti-CD40 (antagonistic), anti-CD40L, anti-B7-1 or anti-B7-2 antibody.

Thus, in another aspect, the invention provides a method for delivering to a mammalian patient, preferably a human patient, multiple administrations of a rAAV virion encoding a therapeutic protein comprising (a) administering to the patient a therapeutically effective amount of rAAV virions encoding the therapeutic protein; (b) prior to, along with immediately after administering the rAAV virion, transiently immunosuppressing the humoral immune system of the patient to obtain expression of the therapeutic protein; and at a later date, repeating steps (a) and (b). Preferably, the immune system of the patient is transiently immunosuppressed both prior to and following the first administration of the rAAV virions. The administering step of the present invention is performed using one of the many conventional techniques known to the art for administering a medicament. Preferably, these techniques include intramuscular administration, intranasal administration, intra-arterial administration and subcutaneous administration.

In yet another aspect, the invention is directed to a pharmaceutical composition comprising in combination an effective amount of an rAAV virion encoding a therapeutic protein, preferably a human therapeutic protein, and an effective amount of an immunosuppressant suitable for transiently immunosuppressing a mammalian patient, preferably a human, in a pharmaceutically acceptable carrier.

In one embodiment, the pharmaceutical composition is in the form of an aerosol suitable for administration by inhalation. The solution may comprise about 10^6 to about 10^{16} particles of rAAV virion and a humoral immunosupressant in a sterile solution of about 0.9% sodium chloride. The aerosol may be contained in a sterile pneumatic aerosol generator reservoir, such that an aerosol of the solution is produced at the rate of about 8 to 12 liters per minute at about 30 to 50 psi of compressed air. In another embodiment, the pharmaceutical may be in lyophilized form, which needs reconstitution with a suitable carrier such as 0.9% saline, D50 water, Ringer's lactate and the like.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and B disclose leptin expression by rAAV vectors. Figure 1A is a representation of the Western Blot analysis of leptin expression *in vitro* from supernatant harvested from human 293 cells infected with $1X10^9$ AAV-leptin (lane 1); $1X10^{10}$ AAV-leptin particles (lane 2); mock infected cells (lane 3); or cells transfected with 2 μg of pCMVKm201-leptin (lane 4). Quantitation of leptin expression for lanes 1-3 of Figure 1A as reported in μg leptin/10^6 cells/day by radioimmunoassay is shown in Figure 1B.

Figures 2A and 2B show the effect of rAAV-leptin treatment on body weight and food intake, respectively, in ob/ob mice which received subcutaneous injections of either 10^{11} particles of rAAV-leptin (O) or saline (Δ) and weights were monitored three times weekly. The mean ±SEM of ten mice in each group was measured. For simplicity in Figures 2A and 2B, only one time point is shown for each week.

Figure 3 is a photograph showing the physical appearance of mice following rAAV-leptin treatment. Mice were photographed six weeks after being treated with rAAV-leptin (left) or saline vehicle (right).

Figure 4 shows the results of measurement of circulating leptin levels (ng/ml) at weeks 5-14 post rAAV-leptin administration in ob/ob mice (dotted bars), relative to ob/ob mice receiving saline (solid bars), and C57 mice (striped bar).

Figures 5A-5C show the results of measurement of the effects of rAAV-leptin on glucose metabolism and insulin secretion in treated and untreated ob/ob mice. Mice were fasted for eighteen hours and bled for determination of fasting glucose (Fig. 5A) and insulin (Fig. 5B), six weeks post-injection. The values presented are the mean ±SEM of five mice. Values are the mean ± of three mice in each group. Tests were performed on fasted mice, eight weeks post-injection.

Figure 6 is a bar graph showing ELISA assay results from Example 4 of erythropoietin ("Epo") expression (mIU/10^6 cells/day) by HT1080 cells infected with rAAV-Epo $5x10^9$ particles; $1x10^9$ particles; $2x10^8$ particles; leptin rAAV (control) and uninfected HT1080 cells (control).

Figures 7A and 7B show the results of *in vivo* administration of rAAV-Epo virions (particles) to mice. Figure 7A is a graph showing the plasma Epo concentration in mIU/ml as a function of time (weeks) in mice administered rAAV-Epo as measured by ELISA. Figure 7B is a corresponding graph showing the hematocrits of four mice administered either rAAV-Epo (squares) or saline (circles)as a function of time (weeks) relative to injection.

Figure 8 shows the results of the ELISA assay using sera from rAAV treated mice and saline treated mice.

Figures 9A and 9B show the results of *in vivo* administration of rAAV-Epo virions (particles) to baboons. Figure 9A is a graph showing the plasma Epo concentration in mIU/ml as a function of time (weeks) in baboons administered rAAV-Epo as measured by ELISA. Figure 9B is a corresponding graph showing the hematocrits of two baboons administered rAAV-Epo as a function of time (weeks) relative to injection.

DETAILED DESCRIPTION

The present invention has multiple aspects. In its broadest aspect, the present invention is directed to a method for obtaining *in vivo* expression in a patient of a therapeutic agent encoded by a gene contained within an AAV vector, the patient suspected of having an immune response to AAV, the method comprising the steps of:

(a) administering to a patient in need of the therapeutic agent a replication defective recombinant AAV particle (virion) having a gene encoding the therapeutic agent; and

(b) before, during or within a short period after administering the AAV vector, transiently immunosuppressing the humoral immune system of the patient to obtain expression of the protein.

In this embodiment, every patient is "suspected of having an immune response to AAV" because the ubiquitous nature of AAV makes it likely that most patients already have an immune response. Thus, rather than prescreen every patient, which would be expensive and dilatory, the vector and a humoral immunosuppressive agent (also referred to herein as "immunosuppressant") are administered in combination to any patient in need of a therapeutic agent.

The method of the present invention is particularly useful when a patient in need of a therapeutic protein requires multiple or ongoing treatments over a period of years. The Applicants' invention allows for multiple administrations to the same patient of the same replication-defective recombinant AAV particles having a gene encoding a therapeutic protein needed by the patient, and provides for expression of the desired recombinant protein even if the patient has developed an immunity to the AAV of the vector particle. Thus, in this second aspect, the invention provides a method for delivering to a mammalian patient, preferably a human patient, multiple administrations of a rAAV virion encoding a therapeutic protein comprising the steps of:

(a) administering to the patient a therapeutically effective amount of rAAV virions encoding the therapeutic protein;

(b) prior to, during or within a short time after administering the rAAV virion, transiently immunosuppressing the humoral immune system of the patient to obtain expression of the therapeutic protein; and

(c) at a later date, repeating steps (a) and (b).

In an embodiment of the above described methods (collectively "method"), the immune system of the patient is transiently immunosuppressed both prior to and following the first administration of the rAAV virions.

The therapeutic agents that are expressed within the method of the present invention include proteins, polypeptides, antisense RNA and ribozymes. Preferably, the therapeutic agents are proteins or polypeptides. Because AAV has a broad cell and host range, the method of the present invention is able to transform the cells of any patient to express one of the above described therapeutic agents therein. The present invention is particularly useful for those patients that require or that would benefit from a therapeutic agent on a continuous or bolus basis.

To determine which arm of the host immune response was responsible for the inability to readminister rAAV vectors, readministration experiments were carried out in class I, class II and CD40 ligand deficient mice as described in Example 9. The results in Example 9 demonstrated that the humoral arm of the immune system played a key role in mounting an immune response to the AAV transfected cells and caused their destruction before the recombinant protein could be expressed. By transiently immunosuppressing the humoral arm of the immune system of the host at the time of first administration of an AAV vector, it is possible to readminister the rAAV virions as described in Example 9. Agents that are used to achieve transient humoral immunosuppression of the patient's immune system include anti-B7-1 or B7-2 antibodies, anti-CD40 (antagonistic) antibodies or CD40 ligand antibodies or a combination thereof.

Methods for making and using many of these antibodies, and antigen binding fragments thereof, are disclosed in U.S. Patent Application Serial No. 08/015,147 (now allowed), U.S. Patent 5,397,703, U.S. Patent No. 5,677,165, U.S. Patent 5,747,034, U.S. Serial No. 08/469,015, U.S. Serial No. 08/463,893, and U.S. Serial No. 08/606,293, all of which are expressly incorporated herein by reference. Other agents, which are useful for transiently immunosuppressing the humoral immune response in a patient, include cyclophosphamide and deoxyspergualin. *See*, Smith, *Gene Therapy 3* (1996) 496-502. Still other agents which are useful to transiently immunosuppress the humoral immune response in a patient include anti-CD3 (OKT3) antibodies, CTLA4Ig (Bristol Myers) and anti-CD4 antibodies and FK506. Preferably, the humoral immune response of a patient is transiently immunosuppressed by administering to the patient a pharmaceutical composition comprising an antibody that is an anti-CD4, anti-CD40 (antagonistic), anti-CD40L, anti-B7-1 or anti-B7-2 antibody, or a combination thereof.

The replication-defective AAV vectors and virions utilized in the method and pharmaceutical compositions of the present invention are prepared using conventional methods of virology, molecular biology, microbiology and recombinant DNA techniques. Such techniques are well known and explained fully in the literature, including, for example, in Sambrook, Molecular Cloning: A Laboratory Manual (Current Ed.); DNA Cloning: A Practical Approach (D. Glover, ed.); Oligonucleotide Synthesis (Current Ed., N. Gait, ed.); Nucleic Acid Hybridization (Current Ed., B. Hames and S. Higgins, eds.); Transcription and Translation (Current Ed., B. Hames and S. Higgins, eds.); CRC Handbook of Parvoviruses (P. Tijessen, ed.); Fundamental Virology, 2d Edition (B.N. Fields and D.M. Knipe, eds.); Current Protocols in Human Genetics, Vol. 1 (N. Dracopoli, ed.). These publications and all other publications referenced throughout this specification are expressly incorporated herein by reference.

Gene transfer, gene therapy or gene delivery refer to methods, techniques or systems for reliably inserting into a host cell a heterologous or a foreign DNA or a DNA not normally expressed. The resultant insertion can be by integration of transferred genetic material into the host cell genomic DNA, by extrachromosomal replication and expression of transferred replicons or in a non-integrated manner.

Vector means any genetic element that is capable of replication when associated with the proper control elements and that can transfer DNA or RNA sequences between cells. Examples include plasmids, phages, transposons, cosmids, chromosomes, viruses, and virions and include cloning and expression vehicles and viral vectors.

The AAV vectors and replication-defective AAV virions utilized herein comprise a DNA encoding a therapeutic protein operably positioned between a pair of adeno-associated virus inverted terminal repeats ("AAV ITRs"). AAV ITRs are art-recognized regions found at each end of the AAV genome that function together in *cis* as recognition signals for DNA replication and for packaging the AAV vector into an AAV coat. The nucleotide sequences of the AAV ITR regions for the various AAV serotypes (*i.e.*, AAV-1 to AAV-7) are known in the art and vary in size with the serotpe. Typically, the AAV ITRs range in size from about 125-145 bp. *See* for example, Kotin, *Human Gene Therapy 5* (1994) 693-801 and Berns "Parvoviridae and their Replication" in *Fundamental Virology,* 2d Edition (B.N. Fields and D.M. Knipe, eds.). As used here, the AAV ITRs of Applicants' recombinant replication-defective retrovirion need not be identical to the nucleotide sequence of the native, *i.e.*, wild-type, sequence, but may be altered by insertion, deletion or substitution of nucleotides. Further, the two AAV ITRs may be derived from any of the AAV serotypes, for example AAV-1, AAV-2, AAV-3, AAV-4, AAV-5 and AAV-7, and need not be identical to or derived from the same serotype, so long as they permit integration

of the heterologous sequence of interest into the recipient cell genome when AAV *rep* gene products are present in the cell.

The AAV *rep* coding region is the art-recognized region of the AAV genome that encodes the proteins required for replication of the viral genome and for insertion of the viral genome into a host genome during latent infection. The *rep* coding region includes at least the four genes encoding the two long forms of *rep* (*rep* 78 and *rep* 68) and the two short forms of *rep* (*rep* 52 and *rep* 40). For more details *see*, for example, Muzyczka, *Current Topics in Microbiol. 158* (1992) 97-129 and Kotin, *Human Gene Therapy 5* (1994) 793-801. The *rep* coding region may be derived from any AAV serotype or from a functional homologue such as the human herpes virus 6 *rep* gene. The region need not include all of the native sequence, but may be altered by insertion, deletion or substitution of nucleotides, so long as the sequence that is present provides for sufficient integration when expressed in a suitable recipient cell. Preferably, the AAV vector and virions utilized in the present invention lack one or more of the *rep* proteins so as to render it replication- defective. More preferably, the AAV vector of the present invention lacks all four of the *rep* proteins.

The AAV *cap* coding region is the art-recognized region of the AAV genome that encodes the capsid or coat proteins, VP1, VP2 and VP3, that package the viral genome. For more details, *see*, for example, Muzyczka, *Current Topics in Microbiol. 158* (1992) 97-129 and Kotin, *Human Gene Therapy 5* (1994) 793-801. The *cap* coding region may be derived from any AAV serotype or from a functional homologue. The *cap* coding region may be altered by insertion, deletion or substitution of nucleotides, so long as the sequence present provide for sufficient packaging when expressed in a suitable recipient cell. Although the *cap* coding region is preferably not included in the AAV vectors and the replication-defective AAV virions employed in the present invention, it needs

to be included in a helper vector that is expressed in a packaging cell that recognizes and packages the ITRs and the gene(s) positioned therebetween.

Thus, the term "AAV vector," as used herein means a vector derived from an adeno-associated virus serotype that includes at least those sequences required in *cis* for replication and packaging, for example, a pair of functional ITRs flanking a heterologous (*i.e.*, non-AAV) nucleotide sequence. With this criterion, any AAV vector of any serotype can be employed in the method of this invention. Examples of vectors for use in this invention are the AAV-2 based vectors disclosed in Srivastava, PCT Patent Publication WO 93/09239 or simply a pair of AAV-7 ITRs having one or more genes operatively positioned therebetween.

The AAV ITRs employed in the vectors and virions of the present invention may be the native (wild-type)AAV ITRs or they may be modified. If the ITRs are modified, they are preferably modified at their D-sequences. The native D-sequences of the AAV ITRs are sequences of twenty consecutive nucleotides in each AAV ITR (*i.e.*, there is one sequence at each end) which are not involved in HP formation. The D-sequences of the ITRs are modified by the substitution of nucleotides, such that 5-18 native nucleotides, preferably 10-18 native nucleotides, most preferably 10 native nucleotides, are retained and the remaining nucleotides of the D-sequence are deleted or replaced with non-native, *i.e.*, exogenous, nucleotides. One preferred sequence of five native nucleotides that are retained is 5′ CTCCA 3′. The exogenous or non-native replacement nucleotide may be any nucleotide other than the nucleotide found in the native D-sequence at the same position. For example, appropriate replacement nucleotides for native D-sequence nucleotide C are A, T and G, and appropriate replacement nucleotides for native D-sequence nucleotide A are T, G and C. The construction of four such AAV vectors is disclosed in United States Serial No. 08/921,467, filed September 2, 1997. Other employable exemplary vectors are pWP-19 and pWN-1, both of which are disclosed in Nahreini, *Gene 124* (1993) 257-62. Another example of

such an AAV vector is psub201 as disclosed in Samulski, *J. Virol. 61* (1987) 3096.

Other suitable AAV vectors are the Double-D ITR vector. Methods for making the double-D ITR vectors are disclosed in U.S. Patent No. 5,478,745. Still other suitable AAV vectors are those disclosed in U.S. Patent No. 4,797,368 (Carter) and U.S. Patent No. 5,139,941 (Muzyczka), U.S. Patent No. 5,474,935 (Chartejee) and PCT Patent Publication WO 94/28157 (Kotin). Yet a further example of an AAV vector employable in the methods of this invention is SSV9AFABTKneo, which contains the α-fetoprotein (AFP) enhancer and albumin promoter and directs expression of the herpes simplex thymidine kinase (TK) gene predominantly in the liver. Its structure and method for making are disclosed in Su, *Human Gene Therapy 7* (1996) 463-70).

The replication-defective AAV vectors are packaged into empty AAV capsids to produce the replication-defective AAV virions helper viruses employed in the methods of the present invention. To package the replication-defective AAV vectors, which are typically one or more genes positioned between a pair of ITRs, one employs a helper construct or helper virus that has AAV-derived coding sequences that function in *trans* to enable AAV replication, and that include the AAV *rep* and *cap* sequences. The helper virus has AAV coding sequences but lacks the AAV ITRs and thus are not packaged in the capsids that are produced. This helper virus then provides for transient expression of the AAV *rep* and *cap* genes missing from the AAV vector. For greater details, including exemplary AAV helper constructs, *see,* for example, Samulski, *J. Virol. 63* (1989) 3822-28; McCarty, *J. Virol* 65 (1991) 2936-45 and U.S. Patent No. 5,139,941. One such AAV helper construct comprises pKS *rep/cap*, which contains the genes encoding the AAV-2 *rep* and *cap* polypeptide sequences. Additional examples of helper viruses, constructs and functions that can be employed include the plasmids pAAV/Ad and pIM29+45 (*see* Samulski, *J. Virol. 63* (1989) 3822-28 and

McCarthy, *J. Virol 65* (1991) 2936-45) and those disclosed in U.S. Patent No. 5,622,856.

Accessory functions and accessory function vectors are non-AAV derived functions and vectors containing sequences encoding such functions upon which AAV is dependent for its replication. Such accessory functions can be derived or obtained from any of the known helper viruses, such as adenovirus, herpesvirus (except herpes simplex virus type-1) and vaccinia virus and include moieties and/or sequences involved in activation of gene transcription, DNA replication, synthesis of *cap* expression products and capsid assembly. *See,* for example, Carter, "Adeno-Associated Virus Helper Functions" in CRC handbook of Parvoviruses, Vol. I (1990) (P. Tijssen, ed.); Muzyczka, *Current Topics in Microbiol. 158* (1992) 97-129; Janik, *Proc. Natl. Acad Sci 78* (1981) 1925-29; Young, *Prog. Med Virol. 25* (1979) 1213 and Schlehofer, *Virology 152* (1986) 110-17.

The heterologous nucleotide sequence(s) that are inserted into the replication-defective AAV vectors and virions of the present invention encode one or more therapeutic agents that include a therapeutic protein, polypeptide, antisense RNA or a ribozyme, or a combination thereof. Typically, the vectors or virions contain from one to two therapeutic agents that are native or non-native to the recipient cell but which have a desired biological or therapeutic effect.

As disclosed above, the heterologous nucleotide sequences that are introduced into the replication-defective AAV vectors and virions of the present invention include a gene that encodes a therapeutic protein or polypeptide, preferably a human protein or polypeptide. Examples of therapeutic proteins and polypeptides that would be suitable for expression in the methods of the present invention include the LDL receptor, Factor VIII, Factor IX, phenylalanine hydroxylase, ornithine transcarbamylase, or α1-antitrypsin; a cytokine, such as interleukin (IL)-1, IL-2 IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14 and IL-15, α-interferon, β-interferon, the γ-interferons, tumor

necrosis factor CD3, ICAM-1, LFA-1, or LFA-3, a chemokine including RANTES 1α, or MIP-1β (*see* Cocci, *Science 70* (1996) 1811-15); a colony stimulating factor, such as G-CSF, GM-CSF and M-CSF; growth factors such as IGF-1 and IGF-2; human hormones such as growth hormone, insulin, calcitonin, prolactin, follicle stimulating hormone, luteinizing hormone, chorionic gonadotropin or thyroid stimulating hormone; any one of the hepatitis genes; thrombopoietin, erythropoietin, or leptin or a combination of the above. The nucleotide coding sequences for these proteins and polypeptides are already known in the art. Even more sequences expressible in the methods and compositions of the invention include Protein S and Gas6, thrombin, Coagulation Factor Xa, acidic fibroblast growth factor (FGF-1), basic FGF (FGF-2), keratinocyte growth factor (KGF), TGF, platelet derived growth factor (PDGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF) and HGF activators, PSA, nerve cell growth factor (NCGF), glial cell derived nerve growth factor (GDNF), vascular endothelial growth factor (VEGF), Arg-vasopressin, thyroid hormones asoxymethane, triodothyronine, LIF, amphiregulin, soluble thrombomodulin, stem cell factor, osteogenic protein 1, the bone morphogenic proteins, MFG, MGSA, heregulins and melanotropin. Preferred proteins include but are not limited to erythropoietin, thrombopoietin (G-CSF), Factor VIII, Factor IX, Factor Xa, human growth hormone, leptin and IL-2, the DNA sequences of which are all known in the art, particularly the human DNA sequences. The *in vivo* expression of two typical proteins, erythropoietin ("Epo") and leptin from rAAV-Epo and rAAV-leptin, respectively, using the methods of the present invention are disclosed in the examples herein.

An antisense sequence that is expressible by the replication-defective AAV vectors and virions of the present invention is an RNA sequence that can prevent or limit the expression of over-produced, defective, or otherwise undesirable molecules by being sufficiently complementary in sequence to the target sequence that binds to the target sequence. For example, the target

sequence can be part of the mRNA that encodes a protein, and the antisense RNA would bind to the mRNA and prevent translation. The target sequence can be part of a gene that is essential for transcription, and the antisense RNA would bind to the gene segment and prevent or limit transcription. For example, group C adenoviruses Ad2 and Ad5 have a 19 kilo Dalton glycoprotein (gp19) encoded in the E3 region of the virus that binds to class 1 MHC molecules in the endoplasmic reticulum of cells and prevents terminal glycosylation and translation of the molecule to the cell surface. Prior to liver transplantation, the liver cells may be infected with gp19-encoding AAV vectors or virions which upon expression of the gp19 inhibit the surface expression of class 1 MHC transplantation antigens. These donor cells may be transplanted with low risk of graft rejection and may require a minimal immunosuppressive regimen for the patient. It may also permit a donor-recipient state to exist with fewer complications.

The ribozymes that are expressed by the replication-defective AAV vectors and virions in the method of the present invention are useful in treating various diseases and conditions. Ribozymes are RNA polynucleotides capable of catalyzing RNA cleavage at a specific sequence and hence useful for attacking particular mRNA molecules. In chronic myelogenous leukemia for example, the "Philadelphia chromosomal translocation" causes expression of a bcr-abl fusion protein and abnormal function of the abl oncoprotein. Because the fusion mRNA occurs only in cells that have undergone the chromosome translocation and because the fusion transcript contains only two possible sequences at the splice junction, a ribozyme specific for either of the two bcr-abl fusion mRNA splice junctions can inhibit expression of the oncoprotein. Exemplary ribozymes include ribozymes to hepatitis A, hepatitis B and hepatitis C. *See* Christoffersen and Marr, *J. Med Chem 38* (1995) 2023-37 and Baarpolome, *J. Hepatol 22* (1995) 57-64. *See* U.S. provisional Patent Application Serial No. 60/025,616 filed September 06, 1996, and herein incorporated by reference.

In another embodiment of the present invention, the protein or polypeptide encoded by the genes inserted into the replication-defective AAV vectors and virions of the present invention provide one or more antigens from pathogenic agents that may be used to immunize the patient. In this embodiment, the rAAV vectors or virions are administered in accordance with the methods of the present invention, but are employed as vaccines as described in U.S. Patent Application Serial No. 09/096,966, filed June 12, 1998 and herein incorporated by reference. Preferred antigens are HCV antigens, such as HCV NS3, NS4, E1, E2 and/or E2a. Also preferred are *H. Pylori* antigens VacA (cytotoxin), heat shock protein, CagA (cai antigen) and urease B. Specific examples of other antigens useful in this invention include HSV (herpes simplex virus), gD, gB and other glycoproteins, HIV gp 120, p24 and other proteins, CMV (cytomegalovirus) gB or gH glycoproteins, hepatitis D virus (HDV) delta antigen, hepatitis A virus (HAV) antigens, EBV (Epstein Barr virus), MMR and VZV (Varicella Zoster virus) antigens, influenza antigens, rabies antigens and bacterial antigens from *Bordetella pertussia*, *Neiserria meningitides* (A, B, C, Y 135). The nucleotide sequences encoding these antigens or antigenically active fragments thereof are well known to those of ordinary skill in the art.

By the term "active fragment," as used herein, is meant a polypeptide containing less than a full-length sequence that retains sufficient biological activity to be used in the methods of the invention. By the term "analog," as used herein, is meant a truncated form, splice variant, mutein with amino acid substitutions, deletions or additions, an allele, or derivative of the mature protein or polypeptide which possess one or more of the native bioactivities of the full length protein or polypeptide. Thus, polypeptides that are identical or contain at least 60%, preferably 70%, more preferably 80% and most preferably 90% amino acid sequence homology to the amino acid sequence of the mature protein wherever derived, from human or non-human sources are included within this definition.

In addition to the AAV ITRs and the above-described nucleotide sequences, the replication-defective AAV vectors and virions employed in the present methods and composition may also include control sequences, such as promoters and polyadenylation sites, selectable markers, reporter genes, enhancers and other control elements permitting for transcription induction and/or selection. The insertion of these sequences and sites is performed using conventional techniques that are well known in the art.

To produce the replication-defective AAV virions of the present invention, the AAV helper construct is used to complement AAV functions missing from the AAV vector which are necessary for the production of AAV virions, in particular, the *rep* and *cap* functions. Suitable helper constructs having complementing functions are well known in the art. The AAV vector, helper construct and adenoplasmid accessory (helper) construct are introduced into the host cell either simultaneously or sequentially, using any of the well known, art recognized transfection techniques, for example by calcium phosphate coprecipitation. Culture conditions include incubation in the range of 33° to 39°C, preferably 37°C for approximately 48 to 120 hours. The cells are collected and lysate produced using three freeze/thaw cycles by sonication. The lysates are then centrifuged to remove cell debris and the rAAV virions purified by cesium chloride equilibrium gradient centrifugation. Any residual adenoviral particles can be inactivated by heating the purified rAAV preparation to at least 56°C for 20-30 minutes. Alternatively, the rAAV virions can be purified by sulfonated cellulose column chromatography following the protocol described in Tamaose, *Human Gene Therapy, 7* (1996) 507-13.

The AAV virions of the invention are employed in pharmaceutical compositions for the treatment of diseases and/or conditions in which systemic administration of a therapeutic substance, for example, a secretory protein is desired or for preventing infections by the organism whose antigen is incorporated into the AAV vector. The pharmaceutical compositions comprising an effective

amount of the AAV virions of the invention in admixture with a pharmaceutically acceptable carrier, for example, a sterile, non-immunogenic solution (such as 0.9% NaCl, D50 water, Ringer's lactate, or phosphate buffered saline) or with an adjuvant/antigen presentation system (such as alum). Other adjuvant/antigen presentation systems, for example, MF59 (Chiron Corp.), QS-21 (Cambridge Biotech Corp.), 3-DMPL (3-Deacyl-Monophosphoryl Lipid A) (RibiImmnochem Research Inc.), clinical grade incomplete Freund's adjuvant (IFA), fusogenic liposomes, water soluble polymers or Iscoms (Immune stimulating complexes) may also be used. A mucosal adjuvant for preparation of intra-nasal formulations as described in PCT Patent Publication WO95/17211, published June 29, 1995 (Biocine Application Number PCT/IB95/00013) is preferably employed.

In another aspect, the present invention is directed to a pharmaceutical composition comprising a therapeutically effective amount of a replication-defective AAV virion having a gene encoding a therapeutic agent, preferably a protein, in combination with an effective amount of a humoral immune suppressant, and in a pharmaceutically acceptable carrier.

The pharmaceutical compositions that carry the vectors and virions of the present methods are administered using conventional techniques known to the art for administering any medicament. Preferably, the pharmaceutical compositions employed in the present invention are administered intranasally, intramuscularly, subcutaneously, intravenously or intraarterially. Formulations and modes of administration are discussed in greater detail below.

Formulations and Modes of Administering AAV Vectors

A. Intranasal Administration

The nasal cavity offers an important route of administration for the recombinant AAV virions of the present invention. The human nasal cavities have a total surface area of approximately 150 cm^2 and are covered by a highly vascular

mucosal layer. A respiratory epithelium comprised of columnar cells, goblet cells and ciliary cuboidal cells line most of the nasal cavity. *See* Chien, *Crit Rev. in Therap Drug Car sys 4* (1987) 67-194. The subepithelia contains a dense vascular network and the venous blood from he nose passes directly into the systemic circulation, avoiding first-pass metabolism in the liver. By avoiding first-pass metabolism, delivery to the upper region of the nasal cavity may result in slower clearance and increased bioavailability. The absence of cilia in this area is an important factor in the increased effectiveness of nasal sprays as compared to drops. The addition of viscosity-building agents, such as methycellulose can change the pattern of deposition and clearance of intra nasal applications. Additionally, bioadhesives can be used as a means to prolong residence time in the nasal cavity. Various formulations comprising sprays, drops and powders, with or without the addition of absorptive enhancers, have been investigated. *See*, for example, Wearley, *Crit Rev in Therap Drug Car Sys 8* (1991) 331-94.

It is advantageous to administer rAAV via the intra-nasal route. Intra-nasal administration is easy and convenient, economical, safe (an overdose is, in most instances, treatable) and does not require medical personnel. the nasal route has been shown to be effective for the administration of a number of molecules due to the extensive network of capillaries located under the nasal mucosa. this facilitates effective systemic absorption and when the drug is administered with absorption promoters, absorption occurs rapidly with high bioavailability (*see* Gizurarwon, *Acta Pharm 2* (1990) 105). However, when adenoviral vectors are administered intra-nasally, cellular, humoral and mucosal CTL responses result. Additionally, it is also advantageous to be able to readminister rAAV via the intra-nasal route.

The preparation of such intra-nasal solutions, having due regard to pH, isotonicity, stability and the like is within the skill in the art. Exemplary formulations of the AAV vector containing lactose, trehalose or mannitol for intramuscular or subcutaneous administration can be prepared by combining one

part 2x formulation buffer to one part purified rAAV The lactose buffer and the trehalose buffer each contains 25 mM trimethamine 70 mM NaC1,2 mg/ml arginine, 10 mg/ml human serum albumin (HSA) and either 100 mg/ml lactose or 100 mg/ml trehalose in a final volume of 100 mls at a pH of 7.4. The mannitol buffer contains 25 mM tromethamine, 35 mM NaCl, 2 mg/ml arginine, 10 mg/ml HSA and 80 mg/ml mannitol in a final volume of 100 ml at a pH of 7.4.

The dosage regimen will be determined by the attending physician considering various factors known to modify the action of drugs such as physician condition, body weight, sex, diet, severity of the condition, time of administration and other clinical factors. Exemplary dosage ranges comprise between 10^3 to 10^{14} particles, preferably 10^6 to 10^{16} particles, more preferably 10^8 to 10^{14} particles, most preferably 10^{10} to 10^{12} particles.

Several types of drug delivery devices for the nasal cavity exist (*see* Chein, *Crit. Rev. Therap. Drug Carr. Sys. 4* (1987) 67). These systems include nasal spray, nose drips, saturated cotton pledget, aerosol spray and insufflator. The meter-dose nebulizer can deliver a predetermined volume of the formulation t the nasal cavity. One such nebulizer is the Ultravent, which is available from Mallinckrodt.

The desired formulation of rAAV virion is placed in the reservoir of the Ultravent pneumatic aerosol generator. The generator is driven with compressed air at about 30-50 psi, preferably 40 psi, generating 10 liters/min (at 40 psi) of aerosol. Using one-way valves, nose clips and mouth piece, this system is closed and all gas is inspired or expired through a filter.

B. Intramuscular Administration

Prior to intramuscularly administering the replication defective rAAV virions, the muscle tissue may be injected with a cell proliferating agent. *See* U.S. Patent 5,593,972. Preferably, the cell proliferating agent is bupivacaine.

The bupivacaine may be injected up to about twenty-four hours prior to injection of the rAAV virions. About 50µl to about 2 ml of 0.5% bupivacaine-HCl and 0.1% methylparaben in isotonic NaCl may be administered to the site where the rAAV virions is to be administered. The cell proliferation agent may be included in the formulation with the rAAV virions. Preferably, 50µl to about 1500µl, more preferably about 1 ml of the agent may be included.

C. Subcutaneous Administration

Any of the above-described formulations are administered subcutaneously using standard techniques known even to technicians in the art. For example, a pharmaceutical composition comprising a therapeutically effective amount of the replication-defective AAV virions of the invention, alone or in combination with a humoral immunosuppressant, and in a pharmaceutically acceptable liquid carrier (*e.g.*, 0.9% sterile saline) is taken up in a sterile syringe with a 22 gauge needle and injected under the skin on the forearm of the patient in need of treatment. Optionally, this administration is followed up with the administration of a one or more doses of a humoral immunosuppressant, using the manufacturers recommendations as a guideline with due consideration for the age, health, sex, and size of the patient.

D. Arterial Administration

The same liquid pharmaceutical compositions as described above are used to administer a dose of the replication-defective AAV vectors or virions to a patient via an artery. The intraarterial administration is performed using standard techniques that are known to the art, including the use catheters, which can be threaded through an artery to deposit the dose at a preferred tissue site. The use of such catheterization techniques are employed extensively in cardiac visualization and are readily available to those skilled in the art. A specific

example of administering the replication-defective AAV virions to a renal artery of a patient is disclosed in Example 10 herein.

In Examples 1 through 4, we generated a rAAV vector carrying the mouse leptin cDNA and demonstrated the ability of this vector to express leptin *in vitro* and *in vivo*. Leptin protein is a satiety factor responsible for controlling food intake in mammals. The *ob/ob* mouse lacks functional leptin; continuous delivery of the recombinant leptin protein corrects the deficiency and leads to weight loss. Example 1 details the construction of the rAAV-leptin construct. Example 2 describes the preparation and titering of rAAV-leptin particles. Example 3 details the *in vitro* analysis of rAAV-leptin and Example 4 discloses the *in vivo* administration of the construct in mice. We demonstrated that intramuscular injection of rAAV vector carrying leptin into *ob/ob* mice, which lack a functional leptin gene, leads to long term correction (>80 days) of all metabolic abnormalities tested, including obesity and diabetes.

It is also interesting to note that the weight loss in the treated animals is much more gradual than noticed in experiments where a bolus or recombinant protein is administered. This is reflective of the kinetics of gene expression by rAAV vectors. Expression of a marker gene from rAAV vectors injected into the mouse muscle gradually increases over a period of 4-6 weeks before stabilizing (unpublished data). In contrast, adenoviral gene delivery results in rapid onset of protein expression, which is extinguished within two weeks, presumably by immune response to adenoviral proteins. *See* Muzzin, *Proc. Natl. Acad. Sci.* USA **93**, 14804-14808.

The invention is further exemplified by the administration of the heterologous sequence encoding monkey erythropoietin (Epo), in mammals. Epo, which is produced in the kidney of mammalian adults is a key hormone involved in regulation of erythrocyte differentiation and the maintenance of a physiological level of circulating erythrocytes (red blood cells). Clinically, Epo is the treatment of choice for anemia associated with chronic renal failure or for the treatment of

thalassemia. The biological effect of Epo gene expression is monitored by determining hematocit levels and the circulating concentration of the hormone is measured standard immunoassay or by ELISA.

EXAMPLE 1
rAAV-Leptin Vector Construction

pKm201CMV is an AAV cloning vector in which an expression cassette, consisting of a CMV immediate early enhancer, promoter and intron and a bovine growth hormone (BGH) polyadenylation site, is flanked by inverted terminal repeat (ITR) sequences from AAV-2. pKm201CMV, was derived from pKm201, a modified AAV vector plasmid in which the ampicillin resistance gene of pEMBL-AAV-ITR (*see* Srivastava, (1989) *Proc. Natl. Acad. Sci. USA 86,* 8078-8082) has been replaced with the gene for kanamycin resistance. The expression cassette from pCMVlink, a derivative of pCMV6c (*see* Chapman, (1991) *Nucleic Acids Res. 19,* 193-198) in which the GBH poly A site has been substituted for the SV40 terminator, was inserted between the ITRs of pKm201 to generate pKm201 CMV. To construct the AAV leptin expression vector pc\CMVAAV-m-leptin, a 511bp fragment, encoding murine leptin cDNA (*see* Giese, (1996) *Molecular Medicine 2,* 50-58) was cloned into the Xba I- Bam H1 sites of pKm201 CMV. In addition to the CMV immediate early promoter/enhancer and intron, the AAV vector contains a post-transcriptional regulatory element (PRE) from hepatitis B virus. The PRE, which increases efficiency of mRNA transport (*see* Huang, *Mol. Cell. Bio. 15,* 3864-3869), was included to increase the size of the vector genome for more efficient packaging. A 579 bp fragment, from the post-transcriptional regulatory element (PRE) region of Hepatitis-B (HBV) (*see* Huang & Yen, (1995) *Mol. Cell. Biol. 15,* 3864-3869) was amplified using the primer set:

5' ACATACGCGTGCTTGCGTGGAACCTTTG 3' (SEQ ID NO: 1)

and

5′ TTGTGGCGCGCCAGCTTATCGATTTCGAACCCG 3′ (SEQ ID NO: 2)

The resulting fragment was digested with *AscI* and *MluI* and inserted into an *MluI* site between the leptin coding region and the GH poly A site. Inclusion of the coding region for mouse leptin into this construct results in a 3.4 kb packageable vector genome. This vector plasmid was packaged using standard methods, and a purified stock of 1.25×10^{12} particles/ml was obtained.

AAV helper plasmid pKS*rep/cap* (encoding *rep* and *cap* protein) was constructed by cloning the AAV-2 genome, without the ITRs (AAV-2 nucleotides 192 through 4493) into pBluescript II KS+ (Stratagene, La Jolla, CA).

EXAMPLE 2
Preparation and Titering of
Recombinant AAV-Leptin Particles

rAAV vectors were produced by a modified transient plasmid transfection protocol. *See* Zhou, (1994) *J. Exp. Med. 179,* 1867-1875. Briefly, human embryonic kidney 293 cells, grown to 60% confluence in a 15 cm dish, were co-transfected with 12.5 μg of helper plasmid pKS *rep/cap* and 12.5 μg of vector plasmid pCMVAAV-m-leptin or pCMVAAV-lacZ using the calcium phosphate co-precipitation method. After 8 hr, transfection medium was replaced with IMDM+10% (fetal bovine serum) FBS containing adenovirus type 5 d1312 at a multiplicity of infection (MOI) of 2. Seventy two hours post-infection, the cells were harvested in HEPES buffer (2.5 ml per dish) and lysed by three cycles of freezing and thawing. The cell lysage was centrifuged at 12,000x g for 20 min to remove cell debris. The packaged rAAV virus was purified through two rounds of cesium chloride equilibrium density gradients to remove any contaminating proteins and heated at 56°C for 45 min to inactivate residual adenovirus particles. For estimation of the total number of vector particles, the vector stock was treated with DNAse I, and encapsidated DNA was extracted with phenol-chloroform,

precipitated with ethanol. Released DNA was compared to a known standard by dot blot analysis.

EXAMPLE 3

In Vitro Analysis of Recombinant AAV Leptin (rAAV-leptin)

10^9 or 10^{10} rAAV-leptin particles were diluted in 2 ml of IMDM+10% FBS and added to 1 x 10^6 human embryonic kidney cells (293 cells) plated at 50% confluence on a 6-well dish. Virus was left on cells for 24 hr. Cells were washed and 2 ml of fresh IMDM+10% FBS was added. Supernatant was collected for Western blot or RIA analysis (24-48 hour post0\-infection). *See* Figs. 1A and 1B.

As a control, supernatant was harvested from cells transfected with 2μg of the pCMVAAV-m-leptin packaging plasmid. For transfections, 2μg of pCMV-AAV-m-leptin plasmid was incubated with 10μl of transfection reagent LT1 (Panvera Inc., Madison WI) and added to $5x10^5$ human embryonic kidney cells (293 cells) seeded on six well dishes. Complexes were incubated with cells for four hours and refed with 2 ml of media. Cell supernatant was collected 48 hr post-transfection and analyzed by Western blot or RIA.

For the Western blot, 10μl of supernatant from infected or transfected cells was mixed with 5μl of 3x Laemmli buffer and boiled to denature proteins. Denatured supernatants were electrophorically separated on 14% SDS-PAGE (Novex, San Diego, CA) and transferred onto nitrocellulose. Blots were probed overnight at 4°C with a 1:5,000 dilution of rabbit anti-leptin antibody (Giese, (1996) *Molecular Medicine 2,* 50-58) in PBS, containing 0.1% Tween and 0.2% nonfat dry milk. Following extensive washing, a horseradish peroxidase conjugate of goat anti-rabbit IgG (Boehringer Mannheim, Indianapolis, IN) was added. Following a one hour incubation and further washing, immunoreactive bands were visualized by chemiluminescence (ECL kit, Amersham, Buckinghamshire, England).

Western analysis, using a rabbit anti-mouse leptin antibody, revealed a single immunoreactive protein in the supernatant from transfected cells and cells infected with 10^{10} particles. The secreted leptin protein migrates at the expected size of 16 kDa. No band was visible in the supernatant from cells infected with 10^{9} particles or mock transfected cells. *See* Fig. 1A.

The level of leptin expression was quantitated using a sensitive RIA. While mock infected cells released no detectable leptin into media, cells infected with 10^{9} and 10^{10} particles released 47 and 290 ng of leptin per 24 hr/10^{6} cells, respectively. *See* Fig. 1B. Interestingly, we found that the packaging capability of the rAAV vector is sensitive to the size of the vector genome packaged. Inclusion of PRE sequence to increase the size of the vector from 2840 to 3430 bp helped in improving the functional titer (data not shown). This result is consistent with the findings of Dong *et al.* (1996) *Human Gene Ther.* 7, 2101-2112, who have demonstrated a direct correlation between genome size and titer of recombinant AAV vectors.

EXAMPLE 4
***In Vivo* Administration of rAAV-Leptin**

Earlier reports using recombinant leptin protein have demonstrated that the protein can be delivered by either i.p. or i.v. routes of administration. Reports which demonstrated the delivery of rodent leptin by gene therapy utilized adenoviral based delivery (i.v.) and the expression of the transgene presumably occurred in the liver. *See* Muzzin, (1996) P*roc. Natl. Acad. Sci.* U.S.A. **93**, 14804-14808 and Chen, (1996) *Proc. Natl. Acad. Sci.* USA **93**, 14878-14882. We administered the rAAV vector by the intramuscular route and monitored food intake and weight gain over a period of ten weeks, as follows.

Twenty four to six week old female C57BL/6J-*ob/ob* mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The weights of ten rAAV-leptin treated mice were compared with ten mice treated with 0.9% saline

vehicle on a weekly basis. Following anesthesia with a mixture of ketamine and xylazine, 50µl of normal saline or normal sale or$1x10^{11}$ rAAV particles was injected into the tibialis anterior (TA) muscle. In some experiments, both legs were injected on two successive days, while in other experiments twice as many particles were injected on one day.

The results are summarize din Figure 2A. The effects of rAAV leptin administration were gradual. Treated mice continued to gain weight for the first three weeks, but at a rate that was significantly less than the saline-treated controls (FIGURE 2A). ($p=.004$ for week 2 and .005 for week 3). During the fourth week following administration of vector, the rAAV-injected mice began to lose weight while the sale-treated mice continued to gain weight. During weeks, 5, 6 and 7, the treated mice continued to lose weight at a relatively constant rate (average of 2.3, 2.4 and 2.0 g/animal/week respectively). Weight loss in the treated mice continued through weeks 8, 9, 10, 11 although the rate of loss declined (1.4, 0.59 and 0.35, 1.02 g/animal/wk respectively). There was a slight increase in the average weight of 0.2g during week 12. The saline-injected mice continued to gain weight from week 5-12, *albeit* at a slightly reduced rate. By week 8, the average weight of leptin treated mice was less than half of the control (saline treated) *ob/ob* mice (25.9 vs. 52.1 g) (Fig. 3). Statistics were calculated using a Mann-Whitney two sample test. Calculations were performed one the InSTAT software program. From week 4 onward the weight difference between the two groups was statistically significant with $p<.0005$. The treated mice were observed to be much more physically active than saline treated *ob/ob* mice.

In sum, in comparison to saline treated mice, rAAV-leptin treated mice gained significantly less weight starting from week 1 until the end of the observation period. Treated animals began losing weight by week 4 and continued to lose weight until week 8 at which time weight began to stabilize. At week 8, the average weight of these animals is much closer to the age matched C57 control mice than to untreated *ob/ob* mice.

Monitoring of food intake was begun in the third week following injection. Pre-weighed standard mouse food was added to cages (containing five mice) each evening and the amount of food consumed was measured the following day. The reduction in food intake of treated mice corresponded with the extent of weight loss. As shown in Figure 2B, at the earliest time points monitored (day 18-23) rAAV leptin treated mice ate a daily average of 3.4g of food per mouse as compared to an average of 5.1 g for saline treated controls. The following week (day 24-29) the mice ate an average 2.9 g of food per day and untreated mice at 4.6 g/day. From week 4 through week 7, the leptin treated mice consistently consumed an average 1.9 g of food/day and by week 9 the consumption was ~2.3 g/day. During weeks 10 and 11 consumption, in the leptin treated mice, plateaued at 2.75g/mouse/day. Throughout this time period (weeks 4-11) the saline controls ate an average of 4.6 g/mouse/day. This steady, low level of food intake in treated mice coincided with a constant, gradual rate of weight loss.

To ensure that the observed weight loss was not due to a side-effect of rAAV injection, a second study was performed. In this experiment, *ob/ob* mice were injected with either the rAAV-leptin vector or an rAAV-β-galactosidase vector as a negative control. The kinetics of weight gain for the β-galactosidase treated mice were identical to the saline treated mice in the initial experiment (data not shown). As in the first experiment, leptin treated mice gained weight more slowly in the early time periods and began to lose weight by week 4 (data not shown).

The level of circulating leptin was measured at 5, 7, 9 and 11 weeks after intramuscular delivery of rAAV-leptin. Blood was collected from isofluorane anesthetized mice by retroorbital bleeds and separated into serum. the levels of circulating leptin were measured using a Lincomouse Leptin RIA Kit (Linco, St. Charles, MO). At week 5, the serum leptin levels from five AAV-leptin treated mice and five saline-treated controls were measured. Serum was collected from mice at the indicated times and leptin levels were measured using

the Linco Ria kit. Values are the average of 5 mice ± SEM. Week 7 mice were fasted for eighteen hours prior to serum collection. Mice tested at weeks 5, 9 and 11 were fed *ad libitum* prior to serum collection. Serum was not collected from C57 mice at week 5.

As shown in Figure 4, at week 5 average leptin concentration for the treated mice was 1.7 ng/ml, with a range of 1.3 - 2.34 ng/ml. The saline treated *ob/ob* mice averaged 1.19ng/ml, with a range of 1.01 - 1.34 ng/ml. This background may be due to reactivity with the truncated leptin protein which is the predicted product of the *ob* mutation. At week 7, the same groups of mice and five C57 control mice were tested. The average serum leptin level of the rAAV-leptin treated mice increased to 3.33 ng/ml (range = 2.9 - 4.56), the saline-treated mice again measured 1.2 ng/ml and the normal C57 mice had serum leptin levels averaging 3.76ng/ml. Serum leptin levels in the rAAV leptin treated mice decreased to 2.46 ng/ml at week 9. Untreated *ob/ob* mice had circulating leptin levels of 65 ng/ml and the wild-type C57 mice had levels of 4.19 ng/ml at this timepoint. At the 11 week timepoint, the leptin concentration was 2.97 ng/ml in treated mice versus 1.03 ng/ml of reactive protein in the untreated *ob/ob* mice. This is again in the range of normal C57 black mice (2.31 ng/ml). P values for treated *versus* untreated are .09, .0005, 007 and .0079 for weeks 5, 7, 9 and 11, respectively.

Thus, ectopic expression of physiologic levels of leptin can prevent onset of obesity. Interestingly, the RIA employed in this study also detects some activity in untreated *ob/ob* mice serum. This might be due to the presence of endogenous inactive leptin secreted in this strain of mice (the *ob* defect is due to premature termination codon in the leptin coding sequence).

The *ob/ob* phenotype is characterized by insulin-resistant diabetes; *ob/ob* mice are hyperglycemic, despite elevated levels of circulating insulin. to determine the effects of leptin gene therapy on diabetes, fasting blood glucose and insulin were measured. Mice were fasted for eighteen hours and bled for

determination of fasting glucose (Figure 5A) and insulin (Figure 5B), six weeks post-injection. The values presented here are the mean ± SEM of five mice. (Figure 5C) Glucose tolerance as determined in saline (Δ), r-AAV-leptin treated (O), or C57(*) mice by measuring blood glucose levels at indicated times after intraperitoneal injection of glucose. Values are the mean ± of three mice in each group. Tests were performed on fasted mice, eight weeks post-injection. At week 6, all five saline treated mice tested were hyperglycemic (Figure 5A). The fasting glucose levels ranged from 168-355 mg/dl (normal = 91 - 129 mg/dl) with an average of 259.2. In contrast, all of the rAAV-leptin-treated mice were within, or slightly below, the normal range with a group average of 91.2 mg/dl and arrange of 74-125 mg/dl. The insulin levels in serum from the fasted mice, were also measured (Figure 5B). All mock-treated animals showed marked hyperinsulinemia, with serum insulin levels between 8 and 20 ng/ml. The average serum insulin concentration for AAV-leptin treated animals was .54 ± .1 ng/ml.

Glucose tolerance tests were performed to measure the ability of AAV-leptin treated mice to clear glucose from circulation. At eight weeks after vector administration, a bolus of 1mg/gm glucose was injected i.p. into fasted mice and blood glucose was monitored over time. In control C57 mice and leptin treated *ob* mice, the level of circulating glucose peaked at 30 minutes and returned to normal within 120 min (Figure 5C). In mock treated *ob/ob* mice, the level of glucose was at least twofold greater than the leptin treated mice at all timepoints. The glucose levels in these mice did not normalize within the three hour time course of the study.

Thus, hyperinsulinemia and insulin resistance could be corrected in leptin treated mice. As demonstrated in Figure 5, at week 6 there was a complete reversal of hyperinsulinemia and hyperglycemia in treated animals. The levels of circulating insulin in treated animals were similar to levels reported for C57 mice (.54 ng/ml *versus* .40 ng/ml). rAAV-leptin treated mice had a normal response to a glucose challenge. At week 8, control *ob/ob* mice failed to correct the

exaggerated hyperglycemia state after a post-fast injection of glucose. In contrast, leptin-treated and age-matched C57BL mice corrected their hyperglycemia. this demonstrates that insulin resistance has been corrected and that these mice are able to properly regulate insulin secretion in response to glucose challenge.

Thus, intramuscular administration of a rAAV vector encoding mouse leptin can lead to total correction of the obese phenotype in *ob/ob* mice. Long-term correction of genetic defect by somatic gene therapy is possible by rAAV based vectors.

EXAMPLE 5
Preparation and *In vitro* Analysis of rAAV-Epo Particles

pKm201CMV is an AAV cloning vector in which an expression cassette, consisting of a CMV immediate early enhancer, promoter and intron, and a bovine growth hormone (bGH) polyadenylation site, is flanked by inverted terminal repeat (ITR) sequences from AAV-2. pKm201CMV, was derived from pKm201, a modified AAV vector plasmid in which the ampicillin resistance gene of pEMBL-AAV-ITR (*see* Srivastava, (1989) *Proc. Natl. Acad. Sci. USA 86,* 8078-8082) has been replaced with the gene for kanamycin resistance. The expression cassette from pCMVlink, a derivative of pCMV6c (*see* Chapman, (1991) *Nucleic Acids Res.19,* 193-198) in which the BGH poly A site has been substituted for the SV40 terminator, was inserted between the ITRs of pKm201 to generate pKm201CMV. To construct the AAV Epo expression vector pCMVAAV-Epo, the Avr II – BglII fragment, which encodes the full length monkey Epo sequence, from the cline pMKE83 (ATCC Accession Number 67545) we cloned into the *Xba* I – *BamH* I sites of pKm201 CMV. In addition to the CMV immediate early promoter/enhancer and intron, the AAV vector contains a post-transcriptional regulatory element (PRE) from hepatitis B virus. The PRE, which increases efficiency of mRNA transport (*see* Huang, *Mol. Cell. Bio. 15,* 3864-3869), was included to increase the size of the vector genome for more

efficient packaging. A 579 bp fragment, from the post-transcriptional regulatory element (PRE) region of Hepatitis-B (HBV) (*see* Huang & Yen, (1995) *Mol. Cell. Biol. 15,* 3864-3869) was amplified using the primer set:

5'ACATACGCGTGCTTGCGTGGAACCTTTG 3' (SEQ ID NO: 1)

and

5' TTGTGGCGCGCCAGCTTATCGATTTCGAACCCG 3' (SEQ ID NO: 2)

The resulting fragment was digested with *As* I and *Mlu* I and inserted into an *Mlu* I site between the leptin coding region and the BGH poly A site. Inclusion of the coding region for mouse leptin into this construct results in a 3.4 kb packageable vector genome. This vector plasmid was packaged using standard methods, and a purified stock of 1.25×10^{12} particles/ml was obtained.

AAV helper plasmid pKS*rep/cap* (encoding *rep* and *cap* protein) was constructed by cloning the AAV-2 genome, without the ITRs (AAV-2 nucleotides 192 through 4493) into pBluescript II KS+ (Stratagene, La Jolla, CA).

Recombinant AAV-Epo particles were produced and analyzed following the protocols in Examples 2 and 3, except that HT1080 cells (human fibrosarcoma cells) maintained in DME+ 10% fetal calf serum (FCS), plated (2×10^5 cells) on a 6 well dish the day before infection were used. The cells were infected with rAAV-Epo at different MOI and 48 hours later supernatant was monitored for Epo using the R&D Quantikine ELISA kit (R&D Systems, Minneapolis, MN). The ELISA results are shown in Fig. 2. Titers of rAAV-Epo are indicated on the X-axis. The lane marked leptin represents background levels of Epo secreted from cells infected with 5×10^9 particles of rAAV-m-leptin. The results show that infection of HT1080 cells with 5×10^9 particles produced 10,800 mIU/10^6 cells/day, which is equivalent to 86.4 ng of Epo per day (1 mIU is equivalent to ~ 8 pg of Epo), while infection with 2×10^8 and 1×10^9 particles led to production of 480 and 2200 mIU/10^6 cell/day, respectively.

EXAMPLE 6
***In Vivo* Administration of rAAV-Epo In Mice**

Seven week old female C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). Recombinant AAV-Epo was administered by injection of 50μl of normal or normal saline containing 2 x 10^{11} rAAV particles into the TA muscle of mice anesthetized with a mixture of ketamine and xylazine as described in Example 4.

Blood (200 μl) was collected by using retroorbital bleeds following isoflurane sedation. Whole blood was used for hematocrit estimation and the separated serum was used for detecting the Epo by ELISA. The results are shown in Figure 7. Panel A shows serum Epo concentration (+/-S.E.M.) following rAAV administration. At all timepoints, the saline injected mice had undetectable levels of serum Epo and are not included in the Figure. Panel B shows the average hematocrit of four mice injected with either rAAV-Epo or saline. Although error bars are included, they are obscured by the plot symbols. The 0 week timepoints represent the average baseline hematocrit and serum Epo concentrations for untreated C57BL/6 mice.

The results indicate that circulating levels of Epo began to rise within one week (36.1 mIU/ml) of injection and reached a peak by week 7 (65 mIU/ml). The biological effect of rAAV-Epo was monitored by measuring the hematocrit. Hematocrit levels closely mirrored the amounts of circulating Epo in the treated mice (*see* Fig. 7B). Hematocrits steadily rose through week 7 at which point red blood cells represent greater than 90% of the blood volume (Figure 7B). Remarkably, the mice maintained this high level hematocrit through week 11 with no apparent deleterious effect.

To determine if the EPO levels were affected by an immune response against the foreign transgene, a bioluminescent ELISA assay was developed to detect antibodies against Epo. Microtiter plates (Dynatech Microlite, Chantilly, VA) were coated with 50μl of EPOGEN® (h-erythropoietin, Amgen,

Thousand Oaks, CA) in a 1μg/ml solution in PBS and incubated overnight at 4°C or for one hour at 37°C. The coated wells were blocked with 1x Aqualite Streptavidin Assay Buffer (Sealite Sciences, Boggard, GA) containing 5% goat serum for one hour at 37°C. The plates were then washed three times with 1x Aqualite Washing Buffer containing 1% goat serum and 3% Tween-20. Diluted serum samples (50μl) were transferred onto coated plates and incubated at 37°C for one hour, then washed six times with washing buffer. Primary antibody (goat anti-mouse gig from Sigma) diluted to 1:1000 was added and incubated for one hour followed by washing six times. Streptavidin Aqualite antibody (1:500) was added to each well and incubated at 37°C for one hour followed by washing six times. The luminescence was triggered by injections of 50μl aliquots of 1x Trigger buffer and the plates were read with a Dynatech ML3000 Luminometer (Chantilly, VA). For saline treated mice, sera was pooled prior to ELISA, sera from rAAV treated mice were measured individually. Positive control (+ control) represents sera from cynomolgous monkey-Epo plasmid DNA injected BALB/c mice which had previously been shown to have anti-cm-Epo antibodies. Titer is defined as the dilution of serum required to reduce the signal to levels obtained in wells containing dilution buffer alone.

The results are shown in Figure 8 while serum samples had an anti-Epo titer of $4x10^5$ titers in rAAV-Epo injected and in saline injected mice were one thousand-fold lower. These results contrast with a recently published report using rAAV to deliver a human Epo DNA, *see* Kestrel, *Proc. Natl. Acad. Sci. 93* (1996) 14082-87. This report demonstrated long term expression of human Epo in BALB/c mice, while in C57BL/6 mice, a decrease in circulating reticulocytes and fatal anemia were observed. In contrast, our results demonstrate long term expression of monkey Epo in C57BL/6 mice following intramuscular delivery. We were unable to detect significant amount of antibodies against monkey Epo in the serum of the C57BL/6 mice.

EXAMPLE 7
In vivo Administration of rAAV-Epo In Baboons

Two one year old female baboons weighing between 3.5 and 4.0 kilograms (150-200 times larger than C57BL/6 mice) were used. Prior to injection, the baboons were monitored for two months to determine the baseline values for circulating serum Epo and hematocrit. Animals were sedated with 10 mg/kg ketamine and 0.5 ml of a 2 x 1012 particles/ml stock of rAAV-Epo was injected into the TA of both legs (tenfold more than mice). As a control, two baboon received similar amounts of rAAV vector encoding β-galactosidase.

2.5 ml of blood was collected from each baboon for performing hematocrit and ELISA assays. The levels of Epo were determined from monkey plasma or mouse serum using the R&D Systems Quantikine kit (R&D Systems, Minneapolis, MN).

The results are shown in Figs. 9A-9B. Fig. 9A shows the plasma Epo levels as measured by ELISA. Fig. 9B shows the hematocrits of two baboons at time pre-injection (negative numbers) and post-injection. To prevent stroke, 34 or 40 ml of blood was removed from Baboon 2 at weeks 11 and 13. As shown in Figure 9A, pre-injection values for serum Epo ranged from 1.5 - 3.3 mIU/ml, with minor week to week variation. One week following the injection, Epo levels had increased to 4.5 mIU/ml for Baboon I and to over mIU/ml for Baboon 2. By week 4, values had increased to 11.8 mIU/ml for Baboon 1 and to 11.3 mIU/ml for Baboon 2. Values peaked at weeks 8-10, at which time Baboon I had circulating levels of 35.9 mIU/ml and Baboon 2 had circulating levels of 41.6 mIU/ml.

Prior to treatment, the hematocrit of Baboon 1 ranged from 35.7 to 40% and the hematocrit of Baboon 2 ranged from 38.7 to 42.3%. Despite the presence of elevated serum Epo levels, the hematocrits of both baboons did not increase significantly during the first week. This probably reflects the lag between exposure to Epo and differentiation of precursor cells into erythrocytes. By week

4, the hematocrits of both baboons had increased above the range seen prior to injection. Hematocrits continued to increase until week 10, at which time both baboons showed at least 25 point increases over their pre-treatment levels. The hematocrit of Baboon 2 exceeded 70%; and thus, this animal was phlebotomized o reduce the risk of thrombosis. Following phlebotomy, in which 34 ml of blood was drawn, hematocrit levels in this monkey continued to rise through week 13. The monkey was against phlebotomized at week 13, only to recover to prephlebotomy levels by week 14. The hematocrit level of Baboon 1 reached a maximum of 61.6 at week 10 and remained at this level between weeks 10 and 16. The hematocrit levels stabilized in Baboon 1 and this stabilization occurred despite high levels of circulating Epo. Animals injected with control rAAV-LacZ virus did not show any increase in serum Epo levels (data not shown).

EXAMPLE 8
Formulation and Intra-Nasal Administration of rAAV Virions

To generate an aerosol containing the rAAV virions carrying Epo, 50 µl of a solution of rAAV-Epo virions at a concentration of 1 x 10^{12} in 0.9% NaCl is placed in the reservoir of a Ultravent nebulizer (Mallinckrodt). The nebulizer is driven at 40 psi with compressed air. The size distribution of aerosol droplets is determined by laser particle-size analysis and the relative proportion of the virion preparation is evaluated by collecting the aerosolized droplets in phosphate buffered saline, pH 7.4, as described in Hubbard, *Proc. Natl. Acad Sci 86,* (1989) 680-684.

Seven week old female C57BL/6 mice are obtained from Charles River Laboratories (Wilmington, MA). After anesthetization with a mixture of ketamine and xylazine and baseline bronchoalveolar lavage fluid, blood, and lung samples are obtained, 50 µl of normal saline containing dosages ranging from 1 x 10^6 to 1 x 10^{12} particles of rAAV-Epo is administered via intra-nasal instillation

using an Ultravent (Mallinckrodt) aerosol nebulizer in accordance with the manufacturer's instructions.

Blood (200µl) is collected by using retro-orbital bleeds following isoflurane edition. Whole blood is used for hematocrit estimation and separated serum is used for detecting the Epo by ELISA.

To determine if the Epo levels are affected by an immune response against this foreign transgene, a bioluminescent ELISA assay can be employed to detect antibodies against Epo. Microtiter plates (Dynatech Microlite, Chantilly, VA) are coated with 50µl of EPOGEN® (h-erythropoietin, Amgen, Thousand Oaks, CA) in a 1 µg/ml solution in PBS and incubated overnight at 4°C or for one hour at 37°C. The coated wells are blocked with 1x Aqualite Streptavidin Assay Buffer (Sealite Sciences, Boggard, GA) containing 5% goat serum for one hour at 37°C. The plates are then washed three times with 1 x Aqualite Washing Buffer containing 1% goat serum and 3% Tween-20. Diluted serum samples (50µl) ate transferred onto coated plates and incubated at 37°C for one hour, then washed six times with washing buffer. Primary antibody (goat anti-mouse IgG from Sigma) diluted to 1:1000 is added and incubated for one hour followed by washing six times. Streptavidin Aqualite antibody (1:500) is added to each well and incubated at 37°C for one hour followed by washing six times. The luminescence is triggered by injections of 50 µl aliquots of 1 x Trigger buffer and the lates are read with a Dynatech ML3000 Luminometer (Chantilly, VA). Titer is defined as the dilution of serum required to reduce the signal to levels obtained in wells containing dilution buffer alone.

For administration in humans, equivalent human dosages by weight should be used, for example from 1×10^7 to 1×10^{16} particles in 50 µl volumes. The published clinical protocol approved by the NHLBI Institutional Clinical Review Subpanel on September 21, 1992, the NIH Biosafety Committee on August 21, 1992, the RAC on December 3, 1992 and the FDA on April 16, 1993 relating to the administration of an adenovirus containing the human CFTR cDNA

and available from the Office of Recombinant DNA Activities, Building 31, Room 4B11, The National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland, USA 20892 may be followed.

Intra-nasal formulations of rAAV virions carrying other heterologous sequences can be made in accordance with the methods disclosed herein, for example rAAV-leptin, and administered and tested as described above and employing art recognized methods.

Such formulations may be administered in humans in a fashion analogous to administration in mice, for example, via intra-nasal instillation using an Ultravent (Mallinckrodt) aerosol nebulizer in accordance with the manufacturer's instructions.

EXAMPLE 9
Transient Immunosuppression To Block Humoral Immune Responses

Typically, after a single intramuscular injection of rAAV vector, transgene expression from a second vector injection is not possible. To determine which arm of the host immune response is responsible for the inability to readminister rAAV vectors, experiments were carried out in class I-, class II- and CD40L-deficient mice. Class I-deficient mice do not develop a normal population of CD 8^{++}T cells and are unable to mount cellular immune responses. (*See* Zijlstra, *Nature 344,* (1990) 742-746). Class II-deficient mice are negative for CD 4^{+} T cells and are defective in humoral immune responses. (*See* Grusby, *Science 253* (1991) 1417-20).

Six week old female C57BL/6 mice were purchased from Charles River Labs (Wilmington, MA). C57BL/6 class I deficient and C57BL/6 class II deficient mice were purchased from Taconic Labs (Germantown, NY). CD40 ligand deficient mice and B129 mice were purchased from Jackson Labs (Bar Harbor, ME).

Vector pAAV-lacZ was constructed by cloning the LacZ expression cassette from pCMV-β (Clontech, Palo Alto, CA) containing the CMV promoter, intron, LacZ and SV40 polyadenylation signal into pEMBL-AAV-ITR. Plasmid pkm201 is a derivative of pEMBL-AAV-ITR in which the ampicillin resistance gene was replaced with the gene for kanamycin resistance. *See* Example 1. Plasmid pAAV-Luc was constructed by cloning an expression cassette containing the CMV promoter/intron, luciferase and the bovine growth hormone polyadenylation signal into pKm201. Plasmid pKS*rep/cap* was constructed by cloning the AAV-2 genome, without the ITRs (AAV-2 nucleotides 192 through 4493) into pBluescript II KS+ (Strategene, La Jolla, CA). *See* Example 1.

Recombinant AAV particles were produced as disclosed in Example 2. Residual adenovirus contamination was inactivated by heating at 56°C for 45 min. To estimate total number of rAAV particles, the stock was treated with DNAse I and encapsidated DNA was extracted with phenol-chloroform and precipitated with ethanol. DNA dot blot analysis against a known standard was used to determine titer. To assay for adenovirus contamination, 293 cells were infected with 10μl of purified rAAV stock and followed for any signs of cytopathic effect. All stocks were negative, indicating that adenovirus contamination was less than 100 pfu/ml.

The rAAV particles were diluted in 0.9% saline and a final volume of 50μl was injected into the tibialis anterior (TA) muscle. On day 0, groups of five class I knockout, class II knockout or C57BL/6 mice were injected with 1 x 10^{10} particles rAAV-LacZ in the right TA. At four weeks after the first injection, the mice were bled for serum and injected with either 1 x 10^{10} particles rAAV-LacZ (three animals) or 1 x 10^{10} particles of rAAV-Luc (two animals) in the left TA. At six weeks, the mice were bled again, sacrificed and muscles were collected and immediately frozen in liquid nitrogen for either LacZ staining or luciferase assay.

For transient immuosuppression by anti-CD4 antibody, mice were injected with 100μg rat anti-mouse CD4 (clone GK1.5, Pharmingen, San Diego, CA) by intraperitoneal injection at days -3, 0 and +3 relative to the first injection (at day 0) of rAAV. For anti-mouse CD40 ligand treatment, mice received 100μg of antibody (clone MR1, Pharmingen, San Diego, CA) by intraperitoneal injection at days -3, 0 and +3 and +6 relative to the first injection of rAAV. Mice treated with cyclosporin A (Sandimmune, Sandoz) received intraperitoneal injections of 10 mg/kg drug every five days from one week before the first injection of rAAV until the termination of the experiment.

For the luciferase assay, the frozen muscles were ground in a pre-chilled mortar and pestle, transferred to a 1.5 ml microfuge tube and resuspended in 500μl 1x reporter lysis buffer (Promega, Madison, WI). The tubes were vortexed for fifteen minutes at room temperature and then freeze/thawed three times. Lysates were cleared by centrifuging at maximum speed in a microfuge for ten minutes and then stored at -80°C until assayed. Luciferase assays were performed using the manufacturer's protocol (Promega, Madison, WI) and read on a Dynatech ML3000 (Chantilly, VA) plate luminometer. Protein concentration of each of the lysates were assayed by BCA protein assay (Pierce, Rockford, IL) and luciferase activities were expressed as picograms of luciferase per mg protein.

Cryosections (8μm) were fixed for five minutes at room temperatures in 10 mM phosphate buffered saline (PBS) containing 1% paraformaldehyde. The fixed sections were stained with X-gal solution (PBS containing 1 mg/ml 5-bromo-4-chloro-3-indoyl-β-galactopyranoside, 1 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, and 5 mM $K_4Fe(CN)_6$) for sixteen hours at 37°C. Sections were counterstained with Nuclear Fast Red.

To perform the AAV capsid ELISA, microtiter plates were coated overnight at 4°C with 10^9 rAAV-LacZ particles/well in PBS. The following day, the plates were washed and then blocked for thirty minutes at 37°C with PBS containing 1% goat serum and 0.3% Tween 20. Serial three-fold dilutions of

sample and control sera were loaded onto the plate starting at 1:75 (control sera was from mice which had received multiple injections of AAV). The microtiter plates were then incubated for one hour at 37°C. Plates were washed and incubated at 37°C for thirty minutes with goat anti-mouse Ig-HRP (immunoglobulin labeled with horseradish peroxidase) at 1:2000 (Dako, Carpenteria, CA). O-phenylenediamine substrate was used to develop the plates. Plates were read at 492 nm with a cut-off of 0.2 OD.

To perform the AAV neutralizing antibody assay, 293 cells were plated at $3x10^4$ cells/well in a 96 well microtiter plate. The following day, pre-bleed, positive control and sample sera were inactivated at 56°C for thirty minutes. Three-fold serial dilutions of sera in IMDM without fetal calf serum (FCS) (Biowhittaker, MD) were then incubated with 10^8 particles of rAAV-Luc at 37°C for one hour. The media was removed from the 293 cells and diluted sera plus virus was added and incubated for one hour at 37°C. After this incubation, the plates were washed, and fresh IMDM containing 10% FCS was added. Twenty-four hours later, cells were rinsed with PBS and lysed in reporter Lysis Buffer (Promega, Madison, WI). Cells were then harvested and assayed for luciferase activity. The AAV neutralizing antibody titer was defined as the dilution of serum required to see 50% of the luciferase activity in 293 cells infected with rAAV-Luc pre-incubated with negative control serum.

The results are shown in Table 1 below in which + indicates 1 - 10%, ++ indicates 10-50%, +++ indicates 50-90% and ++++ indicates 90-100%. NA stands for not applicable.

TABLE 1

rAAV Mediated Transgene Expression After Readministration in Class I and Class II Deficient Mice

Strain And Animal	*Treatment*		*LacZ staining (2 wks post-2d)*		*Luciferase activity (2 weeks post-2d)*	*Anti-AAV titer (time of 2d injection)*	*AAV neutralizing titer (time of 2d injection)*
	1st injctn	*2nd injctn*	*right*	*left*	*left*		
C57BL/6							
1	LacZ	LacZ	++	-	NA	2434	2500
2	LacZ	LacZ	++	-	NA	3355	6000
3	LacZ	LacZ	++	-	NA	3220	3500
4	LacZ	Luc	++++	-	158	5548	5000
5	LacZ	Luc	+	NA	50	2673	5500
Class I Neg							
1	LacZ	LacZ	++++	++	NA	542	900
2	LacZ	LacZ	++++	+	NA	395	700
3	LacZ	LacZ	+++	+	NA	339	750
4	LacZ	Luc	+++	NA	581	580	700
5	LacZ	Luc	+++	NA	825	734	900
Class II Neg							
1	LacZ	LacZ	++++	++++	NA	<75	<15
2	LacZ	LacZ	+	++++	NA	<75	<15
3	LacZ	LacZ	+++	+++	NA	<75	<15
4	LacZ	Luc	++++	NA	2744	<75	<15
5	LacZ	Luc	+++	NA	8450	<75	<15

As shown in Table 1, no luciferase expression was found in C57BL/6 mice previously injected with rAAV-LacZ. High levels of luciferase expression were found in the muscles of Class II deficient mice and intermediate levels were found

in the muscles of Class I deficient mice. Results were similar when the second injection was rAAV-LacZ. Antibody titers to AAV capsids were determined by ELISA and are shown in Table 1 above. The control C57BL/6 mice had high ELISA and neutralizing titers. As expected, the Class II deficient mice did not develop antibody titers, ELISA or neutralizing, against AAV. Antibody titers in the Class I deficient mice were lower than that found in the control mice and resulted in an intermediate level of luciferase or lacZ expression.

To further establish the role of the humoral immune response in readministration, experiments in CD40L (CD40 ligand) deficient mice. CD40L is expressed on activated CD4+ T cells and is critical for their ability to provide help to B cells. (*See* Durie, *Immunol. Today 15* (1994) 406-11; Xu, *Immunity 1* (1994) 423-31 and Yang, *Science 272* (1996b) 1862-67). CD40 ligand deficient mice are known to be deficient in humoral immune responses. An experiment identical to the experiment described above was performed in CD40 ligand deficient mice, except that in all mice the second injection was rAAV-Luc and B129 mice were used as the control. The results are shown in Table 2 below. NA stands for not applicable, and ND stands for not determined.

As shown in Table 2, readministration of rAAV was not possible in the wild type control mice (B129) due to the high anti-AAV titers, but possible in the CD40 ligand deficient mice. The ability to obtain recombinant protein (*i.e.*, luciferase) expression upon vector readministration correlated inversely with anti-AAV antibody titer and AAV neutralizing antibody titer.

TABLE 2

Readministration of rAAV in CD40 Ligand Deficient Mice

Strain And Animal	*Treatment*		*Luciferase activity (time of 2d injection))*	*anti-AAV titer (time of 2d injection)*	*AAV neutralizing titer (time of 2d injection)*
	1st injctn	*2nd injctn*			
B 129					
1	Nothing	Luc	14	ND	ND
2	Nothing	Luc	17	ND	ND
3	Nothing	Luc	NA	3220	3500
4	Nothing	Luc	158	5548	5000
5	Nothing	Luc	50	2673	5500
B 129					
1	LacZ	Luc	0	2515	10,000
2	LacZ	Luc	0	5179	20,000
3	LacZ	Luc	0	502	1500
4	LacZ	Luc	0	316	1500
5	LacZ	Luc	0	3097	7500
CD40L neg					
1	Nothing	Luc	267	ND	ND
2	Nothing	Luc	15	ND	ND
3	Nothing	Luc	105	ND	ND
4	Nothing	Luc	233	ND	ND
5	Nothing	Luc	159	ND	ND
CD40L neg					
1	LacZ	Luc	200	<75	<15
2	LacZ	Luc	533	<75	<15
3	LacZ	Luc	25	<75	<15
4	LacZ	Luc	19	<75	<15
5	LacZ	Luc	20	<75	<15

To determine whether the dose of the first injection affected the efficacy of the second injection, groups of five C57BL/6 mice were injected with escalating doses of rAAV-LacZ in the right tail artery (TA) and then injected with 1 x 10^{10} particles rAAV-Luc in the left TA four weeks after the first injection. The results are shown in Table 3 below. ND stands for not determined.

TABLE 3

Effect of First Injection Dose On the Efficacy of Second Injection

Animal	*Treatment*		*AAV particles 1st injctn*	*Luciferase activity (2 wks post second injctn))*	*anti-AAV titer (time of 2d injection)*	*AAV neutralizing titer (time of 2d injection)*
	1st injctn	*2nd injctn*				
C57BL6						
1	nothing	Luc	none	4195	ND	ND
2	nothing	Luc	none	1488	ND	ND
3	nothing	Luc	none	2000	ND	ND
4	nothing	Luc	none	1386	ND	ND
5	nothing	Luc	none	2212	ND	ND
C57BL6						
1	LacZ	Luc	10^4	1200	101	<15
2	LacZ	Luc	10^4	2520	112	<15
3	LacZ	Luc	10^4	2356	129	<15
4	LacZ	Luc	10^4	2723	74	<15
5	LacZ	Luc	10^4	3194	248	<15
C57BL6						
1	LacZ	Luc	10^5	1606	ND	<15
2	LacZ	Luc	10^5	2272	159	<15
3	LacZ	Luc	10^5	2491	47	<15
4	LacZ	Luc	10^5	1717	144	<15
5	LacZ	Luc	10^5	744	98	<15
C57BL6						
1	LacZ	Luc	10^8	133	103	<15
2	LacZ	Luc	10^8	557	176	<15
3	LacZ	Luc	10^8	89	193	<15
4	LacZ	Luc	10^8	31	168	70
5	LacZ	Luc	10^8	9	439	70
C57BL6						
1	LacZ	Luc	10^{10}	0	1337	70
2	LacZ	Luc	10^{10}	0	4000	35
3	LacZ	Luc	10^{10}	0	2902	250
4	LacZ	Luc	10^{10}	0	659	1000
5	LacZ	Luc	10^{10}	4	3028	2500

As shown in Table 3, the groups that received the lower doses (10^4, 10^5), were as efficiently re-injected as the native controls. These same groups of mice also did not demonstrate neutralizing antibody responses to AAV indicating that the amount of antigen in these doses may have been too low to elicit an immune response. In fact, the right TA muscles of these mice were all negative for lacZ expression (data not shown). The group that received 10^8 particles of rAAV-lacZ mounted a weak antibody response to AV. This lower antibody response resulted in an intermediate level of luciferase expression from the second injection. In this group, one of the two animals with measurable neutralizing antibody titers showed the lowest luciferase expression. The group receiving 10^{10} particles demonstrated a robust antibody response to AV and second administration was not successful.

Based on the results of the previous experiments, we attempted to reduce the host's antibody response to rAAV by transient immunosuppression. Mice treated with anti-CD4 antibody at the time of first injection were able to be re-injected with rAAV-Luc. Formulation and injection protocols were as previously described. All injections were 1 x 10^{10} particles rAAV unless otherwise noted. Luciferase activity was measured and expressed in picograms luciferase per mg protein. The results are shown in Table 4 below. ND stands for not determined.

TABLE 4

EFFECT OF TRANSIENT IMMUNOSUPPRESSION ON TRANSGENE EXPRESSION IN C57BL/6 MICE FOLLOWING READMINSITRATION OF rAAV

Animal	*Treatment*	*Treatment*	*Luciferase activity (2 wks post second injection))*	*anti-AAV titer (time of 2d injection)*	*AAV neutralizing titer (time of 2d injection)*
	1st injctn	*2nd injctn*			
1	Nothing	Luc	107	ND	ND
2	Nothing	Luc	486	ND	ND
3	Nothing	Luc	605	ND	ND
4	Nothing	Luc	220	ND	ND
5	Nothing	Luc	4671	ND	ND
6	Nothing	Luc	800	ND	ND
7	Nothing	Luc	1188	ND	ND
1	LacZ	Luc	28	3908	6000
2	LacZ	Luc	6	9326	2000
3	LacZ	Luc	14	4564	1000
4	LacZ	Luc	5	7014	2500
5	LacZ	Luc	6	1597	1250
αCD4 treated					
1	LacZ	Luc	2	124	800
2	LacZ	Luc	2	43	<15
3	LacZ	Luc	575	121	70
4	LacZ	Luc	317	47	<15
5	LacZ	Luc	3	2932	1500
6	LacZ	Luc	356	<15	<15
7	LacZ	Luc	920	<15	<15
8	LacZ	Luc	772	60	<15
9	LacZ	Luc	1064	<15	<15
10	LacZ	Luc	19	408	550
αCD40 Ligand Treated					
1	LacZ	Luc	94	489	500
2	LacZ	Luc	31	477	900
3	LacZ	Luc	4	2035	1000

Animal	*Treatment*	*Treatment*	*Luciferase activity (2 wks post second injection))*	*anti-AAV titer (time of 2d injection)*	*AAV neutralizing titer (time of 2d injection)*
Cyclo-sporin treated					
1	LacZ	Luc	0	10,911	8,000
2	LacZ	Luc	0	2,497	5,500
3	LacZ	Luc	0	10,394	20,000
4	LacZ	Luc	0	11,827	10,000
5	LacZ	Luc	0	6,777	10,000

Blood was collected from all anti-CD4 antibody-treated mice one day after the last dose of anti-CD4 antibody (α-CD4) and subjected to FACS analysis. All mice showed greater than 99% reduction in the numbers of $CD3^+CD4^+$ T cells (data not shown) and had normal numbers of $CD3^+CD8^+$ T cells. As shown in Table 4, there was some variability in the level of luciferase expression achieved in the α-CD4-treated mice and four of ten showed little or no luciferase expression. As was seen in the previous experiments, the level of luciferase expression correlated inversely with the AAV antibody titers. In the anti-CD40 ligand antibody experiment, only one of three animals was efficiently re-injected, indicating that anti-CD40 ligand antibody treatment alone is probably not an optimal treatment for readministration. Treatment with cyclosporin alone had no effect on the ability to re-inject rAAV. Both the anti-CD4 and anti-CD40 ligand antibody experiments were repeated with similar results.

The results with Class I and Class II deficient animals demonstrates that the humoral arm of the immune system plays a key role in preventing readministration. As the mouse haplotype may affect the capability of readministration, the experiments with Class I and Class II knock-out mice included the appropriate wild-type background mice as controls (C57BL/6). To further establish the role of the humoral arm of the immune system in readministration, experiments were performed in CD40L knock-out mice. CD40L

is expressed on activated $CD4^+$ T cells and is critical for their ability to provide help to B cells. Thus, the CD40L knock out mice should mimic the responses seen in the Class II knock out mice. The results demonstrated that, as in the Class II deficient mice, the CD40L deficient mice could be effectively readministered with the second dose of virus. It is interesting to note that the rAAV mediated transgene expression levels in this strain of control mice (B129) was much lower than in the C57Bl/6 control mice, demonstrating that mouse haplotype can also influence the expression levels mediated by rAAV vectors.

These preliminary *in vivo* results demonstrate that blocking the humoral response during the primary administration of vector will allow efficient readministration.

EXAMPLE 10
Formulation and Direct Administration of rAAV Virions into the Renal Artery

Seven week old female C57BL/6 mice are obtained from Charles River Laboratories (Cambridge, MA). After anesthetization with a mixture of ketamine and xylazine and baseline blood samples are obtained, a mid-abdomen incision is made and the ureter and uretropelvic junction are freed of connective tissues and vascular structures to expose the left renal artery, which is then clamped. An aliquot of 50 µl of a 5% Dextrose solution containing 10^8 to 10^{12} particles of rAAV-Epo or of rAAV-leptin, produced as described in Examples 1-4 above, is directly infused into the left renal artery using a 30 gauge needle within 1 minute. The renal blood flow is then re-established, 5 minutes after injection by removal of the clamp. The incision is closed and the animals allowed to recover. See Lai, *Gene Therapy 4* (1997) 426-31 and Yamada, *J Clin Invest 96* (1995)1230-37.

Three days after injection, blood samples are collected for analysis. Whole blood is used for hematocrit estimation and separated serum is used for detecting the Epo by ELISA.

To determine if the Epo levels are affected by an immune response against this foreign transgene, a bioluminescent ELISA assay can be employed to detect antibodies against Epo. Microtiter plates (Dynatech Microlite, Chantilly, VA) are coated with 50µl of Epogen (Epotin, Amgen, Thousand Oaks, CA) in a 1 µg/ml solution in PBS and incubated overnight at 4°C or for 1 hour at 37 °C. The coated wells are blocked with 1x Aqualite Streptavidin Assay Buffer (Sealite Sciences, Boggard, GA) containing 5% goat serum for 1 hour at 37 °C. The plates are then washed 3 time with 1x Aqualite Washing Buffer containing 1% goat serum and 3% Tween-20. Diluted serum samples (50 µl) are transferred onto coated plates and incubated at 37 °C for one hour, then washed 6 times with washing buffer. Primary antibody (goat antimouse IgG from Sigma) diluted to 1:1000 is added and incubated for 1 hours followed by washing 6 times. Streptavidin Aqualite antibody (1:500) is added to each well and incubated at 37 °C for one hour followed by washing 6 times. The luminescence is triggered by injections of 50 µl aliquots of 1x Trigger buffer and the plates are read with a Dynatech ML3000 Luminometer (Chantilly, VA). Titer is defined as the dilution of serum required to reduce the signal to levels obtained in wells containing dilution buffer alone.

For mice infused with rAAV-leptin, the animals are fasted for 18 hours and blood collected from the tail vein to determine fasting glucose levels. The mice then receive 1 mg/g body weight of a sterile glucose solution by i.p. injection. The mice are anesthetized with isoflurane and blood samples are collected via retroorbital bleeds at 15, 30, 60, 120 and 180 min following the injection. Circulating glucose is measured using the Lifescan One Touch monitor (Life Scan, Milpitas, CA). Insulin levels are measured with the Linco Rat Insulin

RIA kit (Linco Research Immunoassay, St. Charles, MO). Leptin levels are measured with the Lincomouse Leptin RIA kit.

Formulations of rAAV virions for direct injection into the renal artery carrying other heterologous sequences can be made in accordance with the methods disclosed here and administered and tested as described above and employing art recognized methods. Formulations of rAAV virions for human administration via direct injection into the renal artery are made and administered in a manner similar to that described above. Human doses equivalent (by weight) to the doses employed in mice can be used. Administration can be effected by modification of the medical technique of angiography. As is well known in the art, in angiography a catheter is inserted into the femoral artery of the patient and dye injected in order to visualize the kidney. To administer rAAV virions directly into the renal artery in humans, a catheter is inserted into the femoral artery and a pharmaceutical composition comprising a sterile solution containing an effective amount of a rAAV virion in admixture with a pharmaceutically acceptable carrier is injected. The protocol described in Diseases of the Kidney, 5th ed., Ch 14: Diagnostic and Therapeutic Angiography of the Renal Circulation, pages 465-83 (R. Schrier and C. Gottschalk, eds. 1993) can be employed.

All patents, patent publications and scientific publications cited in this specification are hereby incorporated herein by reference. The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

In the Claims:

1. A method for obtaining *in vivo* expression in a patient of a therapeutic agent encoded by a gene contained within an AAV vector, the patient suspected of having an immune response to AAV, the method comprising:

(a) administering to the patient in need of the therapeutic agent a replication-defective recombinant AAV particle (virion) having a gene encoding the therapeutic agent; and

(b) before, during or within a short period after administering the AAV vector, transiently immunosuppressing the humoral immune response of the patient to obtain expression of the therapeutic agent.

2. The method of claim 1, wherein the patient is a human.

3. The method of claim 2, wherein the replication-defective recombinant AAV particle is administered intranasally, intramuscularly, arterially intravenously, or subcutaneously.

4. The method of claim 3, wherein the immune system of the patient is transiently immunosuppressed by the administration of a humoral immunosuppressant that is an anti-CD4 antibody, anti-B7-1 antibody, anti-B7-2 antibody, anti-CD40 (antagonistic) antibody, anti-CD40L antibody, anti-CD3 (OKT3) antibody, cyclophosphamide, deoxyspergulin, CTLA4Ig, FK506 or a combination thereof.

5. The method of claim 4, wherein the humoral immunosuppressant is an anti-CD4 antibody, anti-B7-1 antibody, anti-B7-2

antibody, anti-CD40 (antagonistic) antibody, anti-CD40L antibody, anti-CD3 (OKT3) antibody or CTL4Ig.

6. The method of claim 1, wherein the therapeutic agent is a protein or polypeptide.

7. The method according to claim 2, wherein the protein or polypeptide is erythropoietin, thrombopoietin (G-CSF), Factor VIII, Factor IX, Factor Xa, human growth hormone, leptin or IL-2.

8. The method of claim 7, wherein protein or polypeptide is leptin.

9. The method of claim 7, wherein the protein is erythropoietin.

10. The method of claim 4, wherein the immune system of the patient is transiently immunosuppressed both prior to and following the first administration of the rAAV virions.

11. A method for delivering to a mammalian patient multiple administrations of a rAAV virion encoding a therapeutic protein, comprising:

(a) administering to the patient a therapeutically effective amount of rAAV virions encoding the therapeutic protein;

(b) prior to, along with immediately after administering the rAAV virion, transiently immunosuppressing the humoral immune system of the patient to obtain expression of the therapeutic protein; and

(c) at a later date, repeating steps (a) and (b).

12. The method of claim 11, wherein the patient is a human.

13. The method of claim 12, wherein the replication-defective recombinant AAV particle is administered intranasally, intramuscularly, arterially intravenously, or subcutaneously.

14. The method of claim 13, wherein the immune system of the patient is transiently immunosuppressed by the administration of a humoral immunosuppressant that is an anti-CD4 antibody, anti-B7-1 antibody, anti-B7-2 antibody, anti-CD40 (antagonistic) antibody, anti-CD40L antibody, anti-CD3 (OKT3) antibody, cyclophosphamide, deoxyspergulin, CTLA4Ig, FK506 or a combination thereof.

15. The method of claim 14, wherein the humoral immunosuppressant is an anti-CD4 antibody, anti-B7-1 antibody, anti-B7-2 antibody, anti-CD40 (antagonistic) antibody, anti-CD40L antibody, anti-CD3 (OKT3) antibody or CTL4Ig.

16. The method of claim 11, wherein the therapeutic agent is a protein or polypeptide.

17. The method according to claim 12, wherein the protein or polypeptide is erythropoietin, thrombopoietin (G-CSF), Factor VIII, Factor IX, Factor Xa, human growth hormone, leptin or IL-2.

18. The method of claim 17, wherein protein or polypeptide is leptin.

19. The method of claim 17, wherein the protein is erythropoietin.

20. The method of claim 14, wherein the immune system of the patient is transiently immunosuppressed both prior to and following the first administration of the rAAV virions.

21. A pharmaceutical composition comprising in combination an effective amount of an rAAV virion encoding a therapeutic protein, and an amount of a humoral immunosuppressant suitable for transiently immunosuppressing a humoral immune response in a mammalian patient in a pharmaceutically acceptable carrier.

22. The pharmaceutical composition of claim 21, wherein said mammalian patient is human.

23. The pharmaceutical composition of claim 22, wherein the humoral immunosuppressant is anti-CD4 antibody, anti-B7-1 antibody, anti-B7-2 antibody, anti-CD40 (antagonistic) antibody, anti-CD40L antibody, anti-CD3 (OKT3) antibody or CTL4Ig.

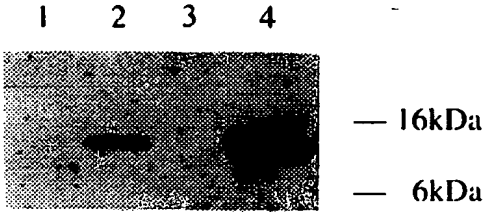

FIG. 1A

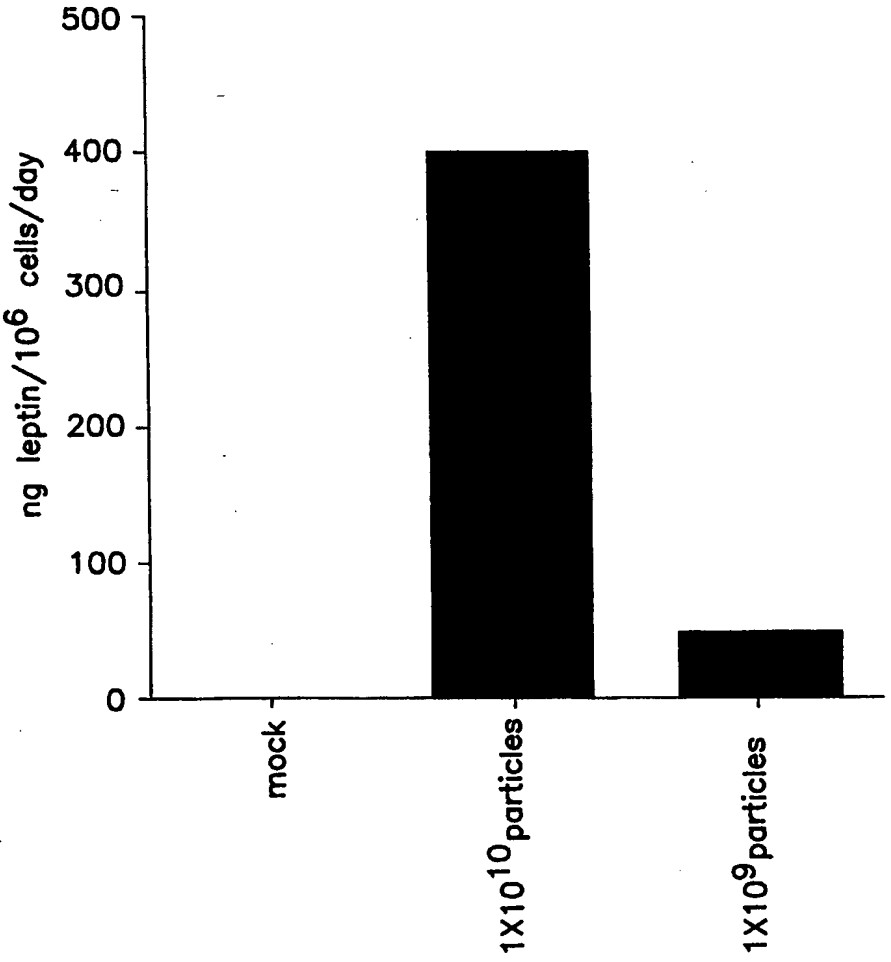
500
400
300
200
100
0
ng leptin/10^6 cells/day
mock
1X10^10 particles
1X10^9 particles

FIG. IB

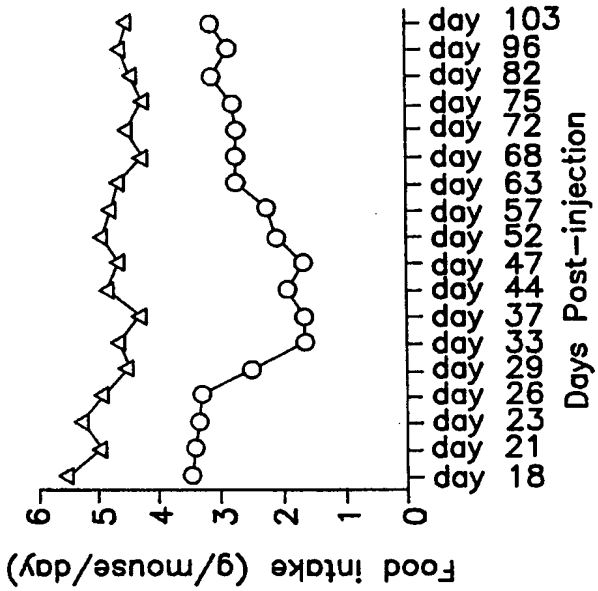
Food intake (g/mouse/day)
0
1
2
3
4
5
6
Days Post-injection
day 103
day 96
day 82
day 75
day 72
day 68
day 63
day 57
day 52
day 47
day 44
day 37
day 33
day 29
day 26
day 23
day 21
day 18

FIG. 2B

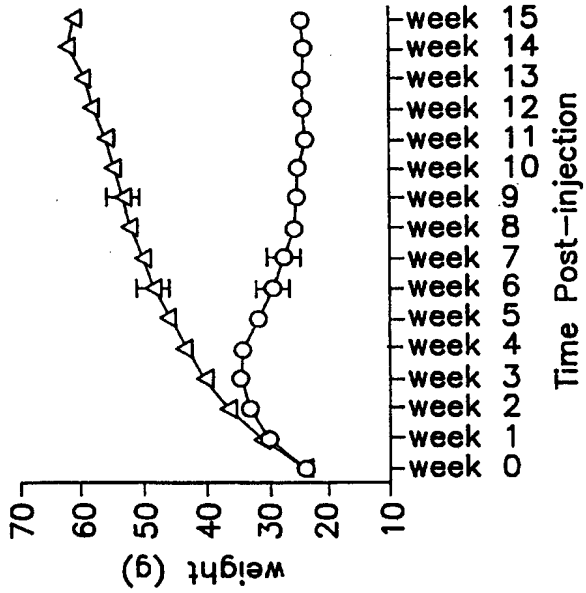
weight (g)
10
20
30
40
50
60
70
Time Post-injection
week 0
week 1
week 2
week 3
week 4
week 5
week 6
week 7
week 8
week 9
week 10
week 11
week 12
week 13
week 14
week 15

FIG. 2A

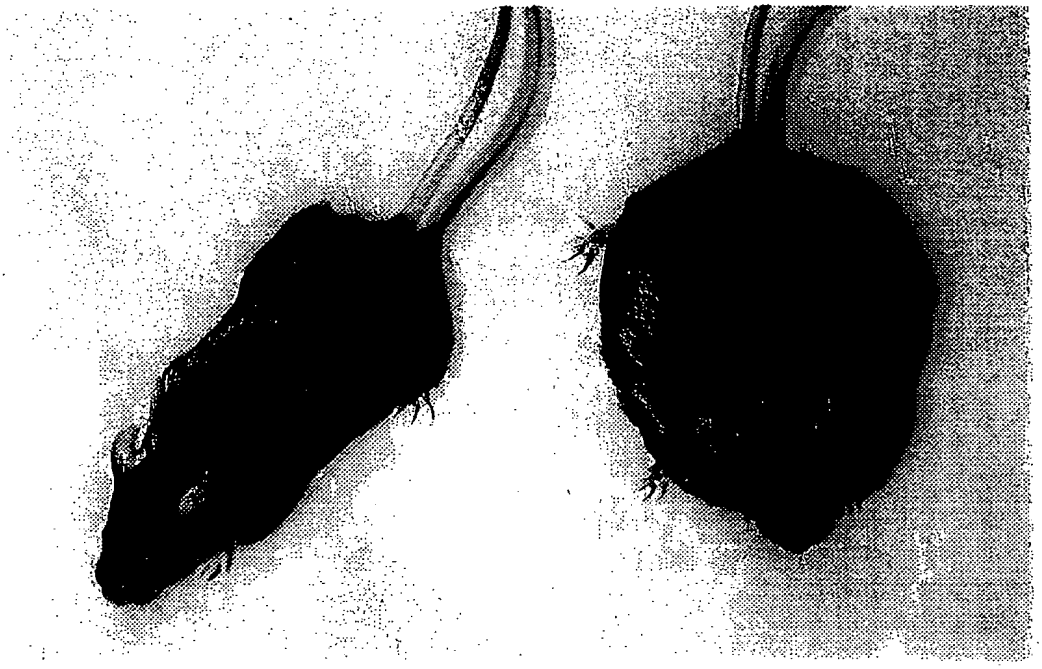

FIG. 3

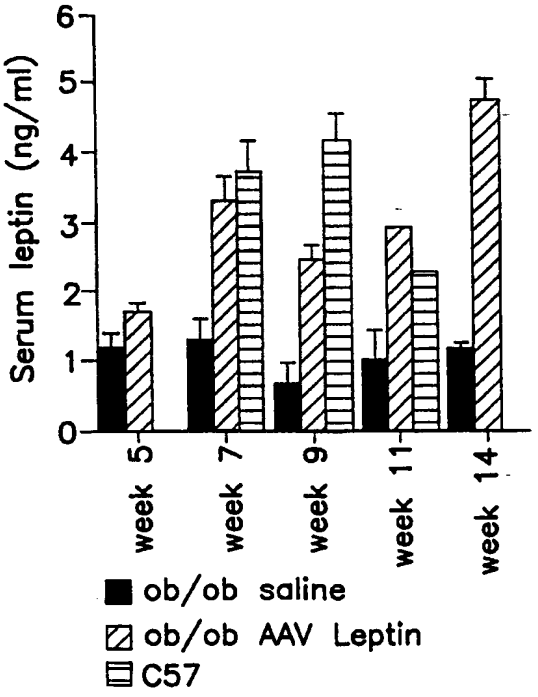
Serum leptin (ng/ml)
0
1
2
3
4
5
6
week 5
week 7
week 9
week 11
week 14
ob/ob saline
ob/ob AAV Leptin
C57

FIG. 4

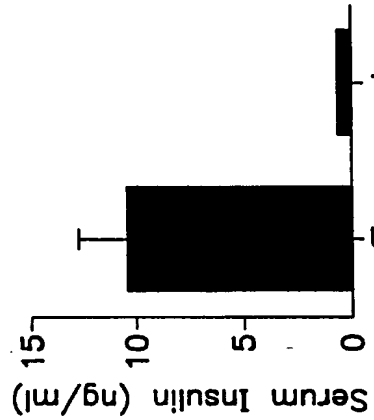

FIG. 5B

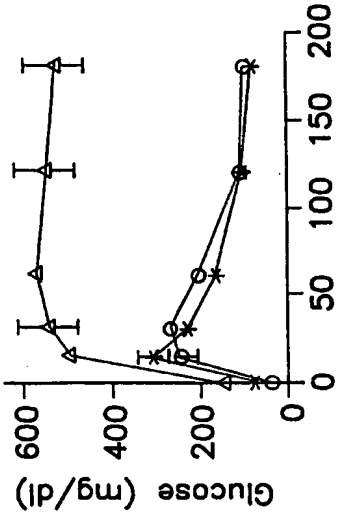

FIG. 5C

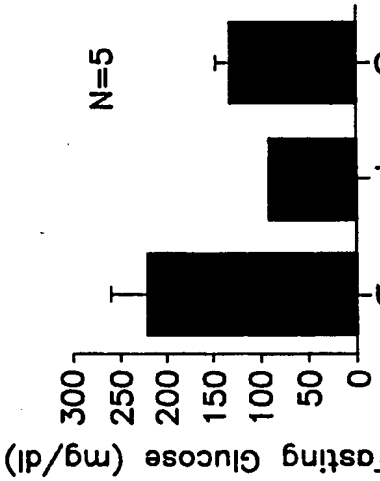

FIG. 5A

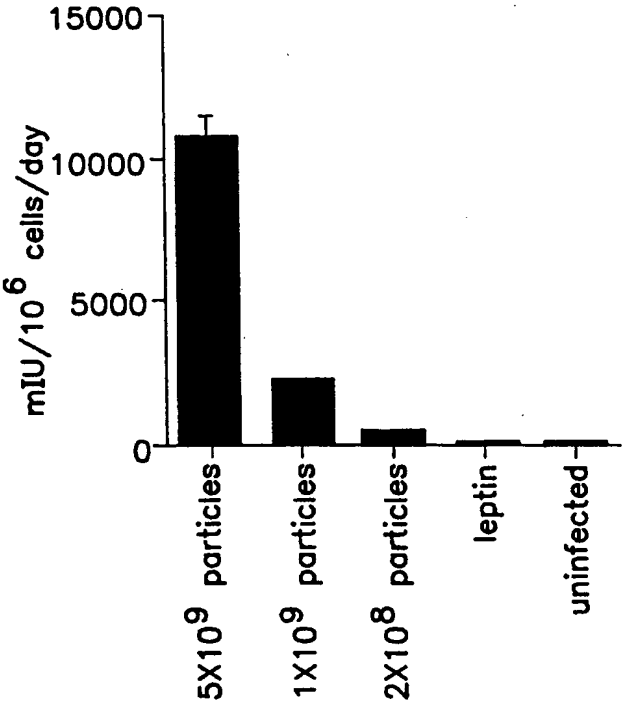
15000
10000
5000
0
mIU/10^6 cells/day
5X10^9 particles
1X10^9 particles
2X10^8 particles
leptin
uninfected

FIG. 6

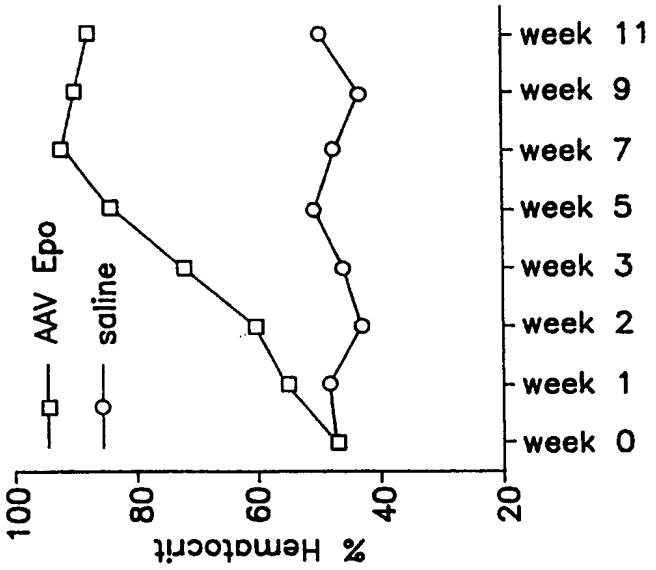
week 11
week 9
week 7
week 5
week 3
week 2
week 1
week 0
AAV Epo
saline
% Hematocrit
100
80
60
40
20

FIG. 7B

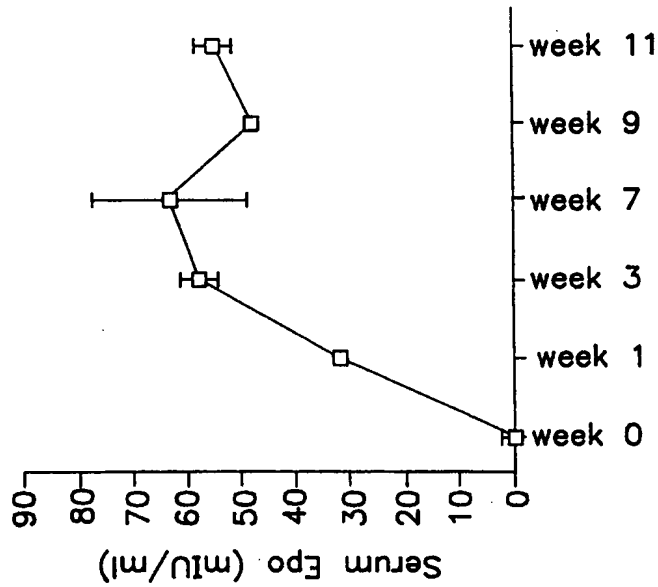
week 11
week 9
week 7
week 3
week 1
week 0
Serum Epo (mIU/ml)
90
80
70
60
50
40
30
20
10
0

FIG. 7A

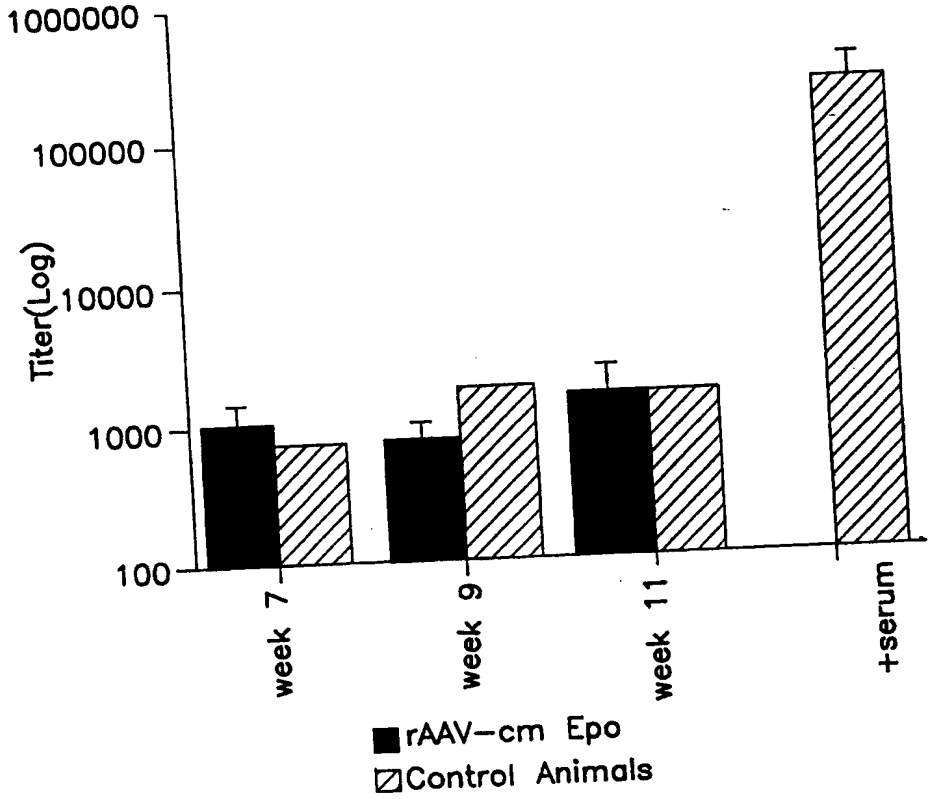
1000000
100000
10000
1000
100
Titer(Log)
week 7
week 9
week 11
+serum
rAAV–cm Epo
Control Animals

FIG 8

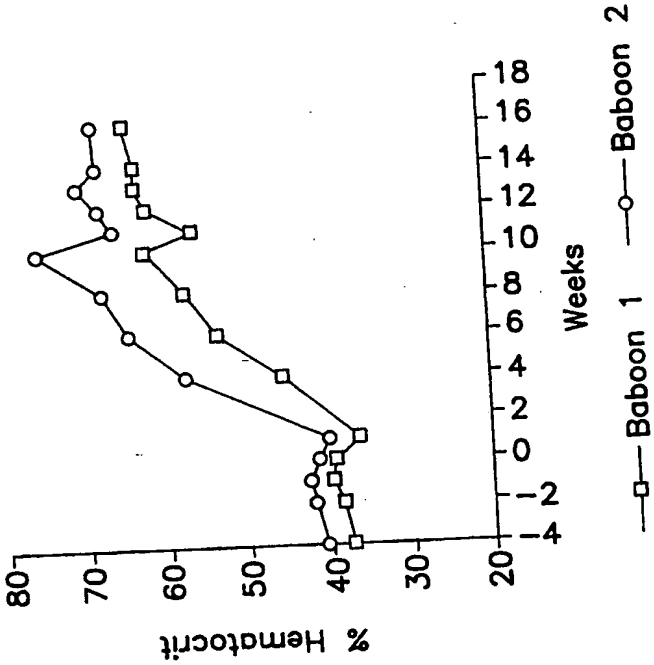
% Hematocrit
80
70
60
50
40
30
20
Weeks
18
16
14
12
10
8
6
4
2
0
−2
−4
Baboon 1
Baboon 2

FIG. 9B

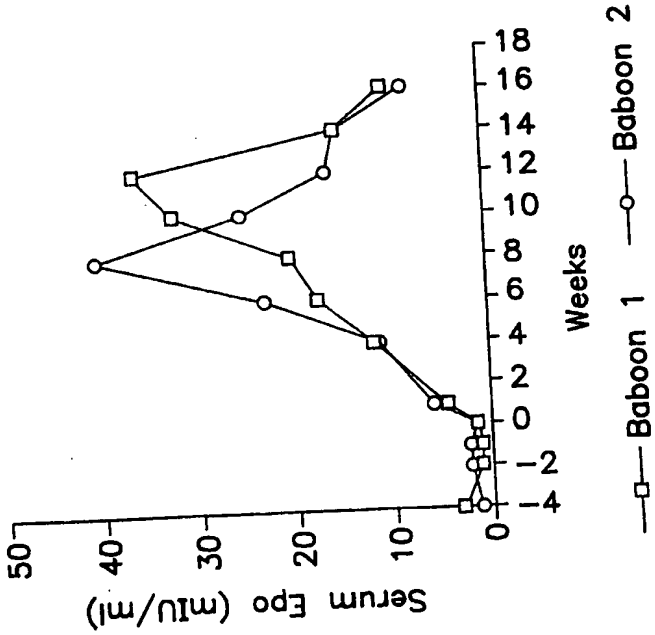
Serum Epo (mIU/ml)
50
40
30
20
10
0
Weeks
18
16
14
12
10
8
6
4
2
0
−2
−4
Baboon 1
Baboon 2

FIG. 9A

SEQUENCE LISTING

<110> Dwarki, Varavani
Zhou, Shangzhen
Murphy, John E.
Manning, William C.
Escobedo, Jaime

<120> METHOD FOR OBTAINING IN VIVO EXPRESSION OF A HETEROLOGOUS GENE CONTAINED WITHIN AN AAV VECTOR

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